ISOLATION AND IDENTIFICATION OF COMPOUNDS CONFERRING PHYTOESTROGENIC ACTIVITY TO *CYCLOPIA* EXTRACTS

by
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Declaration

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Summary

Phytoestrogens are perceived as a safer alternative to conventional hormone replacement therapy (HRT) for the alleviation of menopausal symptoms as they present a decreased side-effect profile. The *Cyclopia subternata* (honeybush) methanol extract, SM6Met, displays estrogenic attributes desirable for the development of an phytoestrogenic nutraceutical, namely, estrogen receptor (ER) α antagonism, ERβ agonism, and antagonism of 17β-estradiol (E_2)-induced breast cancer cell proliferation.

Activity-guided fractionation was employed in an attempt to isolate and identify the compounds inducing the specific estrogenic profile of SM6Met. Fractions were evaluated for estrogenic attributes and major polyphenols present. Initial liquid-liquid fractionation of SM6Met yielded a polar fraction (PF) and a non-polar fraction (NPF), with the estrogenic attributes of interest retained and concentrated in NPF. Subsequent high performance counter-current chromatography (HPCCC) fractionation of NPF yielded three fractions (F1-F3). Interestingly, the fractions revealed separation of the previously demonstrated positive estrogenic attributes of NPF into separate fractions, with F1 and F2 acting as ERα antagonists, only F2 inducing antagonism of E_2-induced breast cancer cell proliferation and only F3 retaining ERβ agonist activity. Although ERβ agonism displayed by F3 was robust and significantly higher than that of 10^{-11} M E_2, it also displayed weak ERα agonism. Fractionation also for the first time in the study revealed ERβ antagonism, as induced by F1. In terms of major polyphenols HPCCC fractionation resulted in a divergence with F1 emerging as the dihydrochalcone-rich fraction and F2 as the flavanone and benzophenone-rich fraction, while the xanthones, flavones and phenolic acids were retained in F3.

In addition, a preliminary absorption study was conducted using the *ex vivo* flow-through diffusion assay whereby the permeability of porcine small and large intestine for polyphenols in SM6Met was evaluated. The major compounds present in SM6Met were not able to penetrate the large intestinal mucosa, but small intestinal permeation of all major compounds in SM6Met ensued, with apparent

permeability coefficient (P_{app}) values ranging from 1.91-3.74 x 10^{-6} $cm.s^{-1}$, indicative of good intestinal absorption. Open source programs used for theoretical prediction of absorption gave conflicting results, emphasising the need to confirm predictions experimentally. ACD/Labs predicted poor intestinal absorption of SM6Met compounds based on physicochemical profiling, while OSIRIS and ChemAxon anticipated good absorption.

In conclusion, activity-guided fractionation results suggest that retention of all the positive estrogenic attributes of the original SM6Met in one fraction is not an attainable goal. This suggests that several of the polyphenols present in SM6Met or NPF, through antagonistic, synergistic, or additive effects, may together be conferring these desired estrogenic traits. Thus production or isolation of a mixture of compounds, i.e. an “intelligent” mixture, should serve as a superior strategy in designing a nutraceutical product tailored to user demand of estrogenic activity.

Opsomming

Fitoestrogene word beskou as ‘n veiliger alternatief vir konvensionele hormoon-vervangingsterapie (HVT) vir die verligting van simptome geassosiaeer met menopause aangesien dit ‘n verminderde newe-effek profiel vertoon. Die metanol ekstrak van *Cyclopia subternata* (heuningbos), SM6Met, vertoon estrogeniese eienskappe wat wenslik is vir die ontwikkeling van ‘n fitoestrogeen nutraseutiese middel, naamlik, estrogeen reseptor (ER) α antagonisme en ERβ agonisme, asook antagonisme van 17β-estradiol (E_2) geïnduseerde proliferasie van borskankerselle.

Aktiwiteit-begeleide fraksionering (ABF) is gebruik om die verbindings wat die spesifieke estrogeniese profiel aan SM6Met verleen te probeer isoleer en identifiseer. Fraksies is ge-evalueer vir estrogeniese eienskappe, asook vir die hoof polifenole teenwoordig. Aanvanklike vloeistof-vloeistof fraksionering van SM6Met het ‘n polêre fraksie (PF) en ‘n nie-polêre fraksie (NPF) opgelewer met behoud en konsentrering van die wenslike estrogeniese eienskappe in NPF. Daaropeenvolgende hoë werkverrigting teen-vloei chromatografie (HPCCC) van NPF het drie fraksies (F1-F3) opgelewer. Interessant genoeg het hierdie fraksies ‘n verdeling van die wenslike estrogeniese eienskappe van NPF in die individuele fraksies teweeggebring, deurdat F1 en F2 ERα antagonisme getoon het, F2 E_2-geïnduseerde proliferasie van borskankerselle antagoniseer het, en net F3 ERβ agonis-aktiwiteit behou het. Alhoewel die ERβ agonis-aktiwiteit van F3 betekenisvol hoër was in vergelyking met die aktiwiteit van 10^{-11}M E_2, het dit ook swak ERα agonisme getoon. Verder het fraksionering, vir die eerste keer in hierdie studie, ERβ antagonisme meegebring soos getoon deur F1. HPCCC het ook ‘n skeiding van die hoof polifenole veroorsaak, waarvolgens F1 as die dihidrogalkoon-ryke fraksie, F2 as die bensofenoon-ryke en flavanoon-ryke fraksie, en F3 as die xantoon-, flavoon- en fenoliese suur-ryke fraksie tevoorskyn gekom het.

‘n Voorlopige absorpsie studie, wat gebruik gemaak het van die *ex vivo* deurvloei diffusie toetssisteem, is uitgevoer om die deurlaatbaarheid van vark dik- en dunderm vir SM6Met polifenole te evalueer. Die hoof verbindings van SM6Met kon nie die dikderm mukosa penetreer nie, maar die

deurlaatbaarheid van die dunderm vir alle SM6Met hoof polifenole is aangetoon, met skynbare deurlaatbaarheidskoëffisiënt (P_{app}) waardes wat strek vanaf 1.91 tot 3.74 x 10^{-6} $cm.s^{-1}$, ooreenstemmend met goeie intestinale absorpsie. Oopbron programme, wat gebruik is vir die teoretiese voorspelling van absorpsie deur gebruik te maak van fisiese-chemiese profilering van verbindings, het teenstellende resultate opgelewer wat daarop dui dat hierdie voorspellings eksperimenteel bevestig moet word. ACD/Labs, wat van die fisiese-chemiese eienskappe van die verbinding gebruik maak, het swak intestinale absorpsie van die polifenole voorspel, terwyl OSIRIS en ChemAxon goeie absorpsie voorspel het.

Ten slotte, resultate van aktiwiteit-begeleide fraksionering het getoon dat behoud van al die gewensde estrogeniese eienskappe van SM6Met in een fraksie nie ‘n haalbare doelwit is nie. Hierdie bevinding dui daarop dat verskeie van die polifenole teenwoordig in SM6Met of NPF saam, deur middel van antagonistiese, sinergistiese of additiewe effekte, die wenslike estrogeniese eieskappe verleen. Die ontwikkeling of isolering van ‘n mengsel van verbindings, met ander woorde‘n “intelligente” mengsel is dus ‘n beter strategie vir die ontwerp van ‘n estrogeniese nutraseutiese produk om die verbruikers-aanvraag van estrogeniese aktiwiteit te voorsien.

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Alphabetical list of abbreviations

15α-OHAD	15α-Hydroxyandrostenedione
15α-OHDHEA	15α-Hydroxydehydroepiandrosterone
15α-OHE_2	15α-Hydroxyestradiol
16α-OHAD	16α-Hydroxyandrostenedione
16α-OHDHEA	16α-Hydroxydehydroepiandrosterone
16α-OHDHEAS	16α-Hydroxydehydroepiandrosterone sulfate
AF	Activation function
AIB1	Amplified in breast cancer-1
Akt	Protein kinase B
ANOVA	Analysis of variance
AP-1	Activating protein 1
AR	Androgen receptor
BCRP	Breast cancer resistance protein
BPI	Base peak intensity
BSA	Bovine serum albumin
C/EBPβ	CCAAT enhancer binding protein β
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyltransferase

CBP	CREB binding protein
CHD	Coronary heart disease
CREB	cAMP response element binding protein
CYP3A4	Cytochrome P450-dependent mixed-function oxidase 3A4
DAD	Diode array detector
DAPI	4'6-diamidino-2-phenylindole-2HCl
DBD	DNA binding domain
DDT	Dichloro-diphenyl-trichloroethane
dH_2O	Distilled water
DHEAS	Dehydroepiandrosterone sulfate
DME	Dried methanol extract
DMSO	Dimethylsulfoxide
E_1	Estrone
E_2	17β-estradiol
E_3	Estriol
E_4	Estetrol
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase

ER	Estrogen receptor
ERE	Estrogen response element
ESI	Electrospray-ionization
F3M	Reconstituted F3 major compound mixture
FCS	Fetal calf serum
G3P	Glyceraldehyde 3-phosphate
GAE	Gallic acid equivalents
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
GRIP1	Glutamate receptor-interacting protein 1
HBA	Hydrogen bond acceptors
HBD	Hydrogen bond donors
HPCCC	High-performance counter-current chromatography
HRT	Hormone replacement therapy
HSCCC	High speed counter-current chromatography
HT	High throughput
IAM	Immobilized artificial membrane
IGF-I	Insulin-like growth factor I
IU	International unit

LBD	Ligand-binding domain
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LDL-R	Low density lipoprotein receptor
MAPK	Mitogen activated protein kinase
MCT1	Monocarboxylate transporter 1
MDCK	Madin-Darby canine kidney
MMP9	Matrix metallopeptidase 9
MR	Mineralocorticoid receptor
MRP	Multidrug resistance-associated protein
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yI)2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor κB
NMR	Nuclear magnetic resonance
NPF	Non-polar fraction
OATP-B	Organic anion-transporting polypeptide B
OCTN2	Organic cation transporter 2
O-DMA	*O*-desmethylangolensin
PAGE	Poly-acrylamide gel electrophoresis

PAMPA	Parallel artificial membrane permeability assay
P_{app}	Apparent permeability coefficient
PBS	Phosphate buffered saline
PEPT1	Peptide transporter 1
PF	Polar fraction
PGC-1	PPARγ coactivator-1
P-gp	P-glycoprotein
PI	Phosphoinositol
PMS	Phenazine methosulfate
PR	Progesterone receptor
PRA	Progesterone receptor isoform A
PRB	Progesterone receptor isoform B
pS2	Position-specific antigen 2
PSA	Polar surface area
PTK	Protein tyrosine kinase
PuF	Purification factor
qHPLC	Quantitative high performance liquid chromatography
qPCR	Quantitative polymerase chain reaction
QSPR	Quantitative structure-property relationship

QTOF	Quadrupole time of flight
R,R-THC	R,R enantiomer of 5, 11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol
RALs	β-resorcylic acid lactones
RB	Rotating bonds
RE	Response element
RLU	Relative light units
RP-HPLC	Reverse phase high-performance liquid chromatography
RPM	Revolutions per minute
SA	Specific activity
SERD	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator
Sp1	Specificity protein 1
SRA	Steroid receptor RNA activator
SRC-1	Steroid receptor coactivator 1
StAR1	Steroidogenic acute regulatory protein 1
TBST	Tris buffered saline plus Tween
TFC	Total flavonoid content
TPC	Total polyphenol content
UPLC	Ultra-performance liquid chromatography

UV	Ultraviolet
VI	Number of Lipinski “rule of 5” violations
WST	Water soluble tetrazolium
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

To

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Chapter 1
Introduction

Nutraceuticals have become a multi-billion dollar industry in the global marketplace with sales in the USA alone reported to be $57.2 billion in 2012 [1]. In 2004 the major contributors to the global market were USA, Japan, and Europe, comprising an estimated 90% of the total market [2]. Defined as non-toxic food extract supplements, with health benefits likely arising from biochemical and cellular interactions [3], nutraceuticals are believed to have fewer side-effects than synthetic drugs [4]. Consumers have shown increased interest in nutraceuticals as a consequence of increased public health consciousness, increasing health costs of modern medicines [5], and also as nutraceuticals are seen as 'safer' alternatives to conventional therapies [6]. Africa is currently not seen as a competitive market for nutraceuticals, however an emerging market is establishing itself in South Africa [7,8], which opens the door for the development of a uniquely South African nutraceutical.

Cyclopia species (family Fabaceae) are endemic to the Western and Eastern Cape provinces of South Africa [9], and the aerial parts of the bush have been harvested for more than a century for the production of a herbal tea known as honeybush tea [10], produced from oxidised plantmaterial. Re-discovery of honeybush in the 1990's led to the production of green or unfermented honeybush, in order to prevent oxidative breakdown of potentially health-promoting polyphenols, as this transpires in the case of fermented honeybush [11]. As consumers world-wide demonstrated an increased demand for health-promoting foods, and especially antioxidants, the export market for honeybush grew from 50 to 200 tonnes from 1999 to 2006 [9]. In 2010, the major importers were the Netherlands, Germany, USA, and the United Kingdom (UK) [9] and at this stage several *in vitro* and *in vivo* studies had already shown that *Cyclopia* species had antimutagenic, antioxidant, anticancer and also phytoestrogenic properties [10].

Menopausal women have shown great interest in phytoestrogenic nutraceuticals as an alternative to conventional hormone replacement therapy (HRT) due to the extensive side effect profile of HRT [12-24], with breast cancer as a primary adverse outcome [23,24]. Initially, epidemiological studies

found a correlation between the consumption of phytoestrogen-rich food and a lowered incidence of breast, prostate, and endometrial cancer, as well as cardiovascular disease [25,26]. More recent studies, however, have shown that known phytoestrogens can not only alleviate menopausal symptoms, but they can also aid in the prevention of numerous diseases such as osteoporosis and cardiovascular disease, and also hormone dependent cancers, such as amongst others, breast cancer [27-35].

The possibility of *Cyclopia* as a phytoestrogen source was first raised in two studies by Verhoog *et al*. [36,37] in 2007. In the initial study [36] it was found that extracts from both *C. genistoides* and *C. subternata* displayed phytoestrogenic activity, and it was also shown that optimal extract estrogenicity was generated by using methanol extracts from unoxidised plantmaterial rather than fermented plantmaterial. In the second study [37] a dried methanol extract (DME) of *C. genistoides*, P104, was identified as binding the estrogen receptor (ER), transactivating an estrogen response element (ERE) containing promoter reporter construct predominantly via ERβ, and also antagonising 17β-estradiol (E_2)-induced breast cancer cell proliferation. Subsequent screening of extracts produced from *C. genistoides* and *C. subternata* species for extraction-enhanced estrogenic activity in 2008 by Mfenyana *et al*. [38], resulted in the identification of SM6Met, a sequential methanol extract of a *C. subternata* harvesting, M6. Mfenyana *et al*. [38] also demonstrated that SM6Met was estrogenically the most potent of the screened *Cyclopia* extracts, with a potency comparable to commercially available phytoestrogenic nutraceuticals. More recently, investigation into the estrogenic specificity of SM6Met revealed that the extract displays three desirable estrogenic traits for future development of a possible phytoestrogenic nutraceutical, namely, ERα antagonism, ERβ agonism, and also antagonism of E_2-induced breast cancer cellular proliferation [39]. This suggested that SM6Met contains a compound or compounds conferring subtype specific estrogenic activity [40], which could play a key role in the development of a future nutraceutical, as it has been shown that ERα antagonism results in the inhibition of cellular proliferation, whilst ERβ activation inhibits ERα mediated breast cancer cell proliferation [41]. A phytoestrogenic

nutraceutical with these traits would thus alleviate menopausal symptoms through ERβ activation, whilst as a positive side effect it would prevent the development of breast cancer through ERα antagonism.

For the future development of a possible phytoestrogenic nutraceutical, the candidate formulation, as for any new nutraceutical, should meet important prerequisites for the marketing of health claims. An important prerequisite is formulation standardisation, which includes efficacy, quality and safety data, however, equally important prerequisites include identification of the active compound(s), and elucidation of the molecular mechanism of action, which covers amongst others absorption, distribution, metabolism and excretion of the active compound(s) [42-45]. Identification of active compounds is not only important for the manufacturing process of a quality-assured phytoestrogenic nutraceutical, but it will also prove to be important in screening and selective breeding of *Cyclopia* plants for effective isolation and/or purification of these compounds.

The remainder of this dissertation consists of four chapters. Chapter 2 provides a literature review discussing the importance of E_2, sources of E_2, its molecular mechanism of action, and its use in conventional HRT. The literature review also includes a section on E_2-associated diseases, and why most phytoestrogens are perceived as safer alternatives to conventional HRT. Furthermore, the literature review includes a section on *Cyclopia*, followed by a section discussing techniques available in identifying estrogenic compounds from an extract. Lastly, the literature review will focus on the intestinal absorption and metabolism of known phytoestrogens and *Cyclopia*-associated polyphenols, and also techniques available to evaluate intestinal permeation of compounds. The literature review aims to validate our decision of identifying and isolating compounds conferring phytoestrogenic activity to *Cyclopia* extracts and establishing whether these compounds are intestinally absorbed. In addition, the literature review aims to enlighten the reader about the potential of phytoestrogens, and *Cyclopia* extracts as a potential source of phytoestrogenic compounds. Chapters 3 and 4 will each address one of our main aims. Chapter 3 focusses on the

isolation of compounds conferring specific phytoestrogenic activity to SM6Met, using activity-guided fractionation of SM6Met combined with two *in vitro* assays to monitor estrogenic activity. The two *in vitro* assays to be used are a cell proliferation assay investigating the effect of fractions on breast cancer cell proliferation, and a promoter reporter assay investigating ERα/ERβ transactivation via an ERE-containing promoter reporter construct. In an attempt to predict human intestinal absorption of SM6Met compounds, chapter 4 investigates small and large intestinal permeation of SM6Met compounds using an *ex vivo* flow-through diffusion assay. Chapter 5 integrates and contextualises the obtained results in terms of the available literature, and discusses how these results fit into the 'bigger picture'. Furthermore, chapters 3 and 4 are written in manuscript format, and as such, each contains an introduction, materials and methods, results, and discussion section, and therefore the occurrence of repetition between chapters are inevitable. We, however, have found the outcomes of the current study to be of a rewarding nature, and in sharing this we hope to enrich the scientist in the reader.

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Chapter 2
Literature review

2.1 Introduction

Menopause is characterised by a decrease in estrogen production from the ovaries due to a decrease in ovarian function [1]. Menopausal symptoms may be divided into climacteric symptoms, such as night sweats, and psychosomatic symptoms such as poor concentration and memory loss [2], however, long term problems such as osteoporosis, Alzheimer's disease and coronary heart disease (CHD) are also common [3-5].

To supplement the deficit in estrogen, menopausal symptoms are traditionally treated with HRT. Conventionally, estrogen or estrogen and progestin are used in HRT, however, estrogen supplementation has been linked to an increased risk of various diseases, amongst others [6-9], cardiovascular disease [9-11] and also various cancers [9,10,12-18], of which breast cancer is considered the primary adverse outcome [9,10].

As a result of adverse effects women have become reluctant to make use of traditional HRT [19] and this has consequently initiated the search for a safer alternative. Phytoestrogens were subsequently identified as a safer alternative [20] as these plant constituents are structurally and or functionally similar to mammalian estrogen [21]. Phytoestrogens, however, present a subtle uniqueness in their mechanism of action that not only supplements the estrogen deficit in menopausal women, but as a side effect may result in the prevention of certain hormone-dependent cancers [22,23], such as hormone dependent breast cancer.

Studies have shown that *Cyclopia* species, better known as honeybush, serve as a possible phytoestrogen source [24-26]. *Cyclopia* forms part of the fynbos kingdom of the Western and Eastern Cape provinces of South Africa [27], and has traditionally been marketed and consumed as a herbal infusion. In addition to findings supporting *Cyclopia* as a possible phytoestrogen source, a specific *C.subternata* extract was shown to be comparable to commercially available phytoestrogenic nutraceuticals in terms of its estrogenic potency [26]. Subsequent estrogenic specificity evaluation of this extract in a recent review identified three estrogenic attributes

desirable for the development of a possible phytoestrogenic nutraceutical [28], which will be discussed in this review.

Prerequisites in the development of an oral phytoestrogenic nutraceutical from a plant extract include, amongst others, identification of the active compounds, and also whether these compounds can become bioavailable in the human body in order to exert a desired therapeutic effect [29-32]. The most obvious choice in the isolation and identification of desired active compounds from extracts is activity-guided fractionation, as this technique is most frequently and successfully employed for this purpose [33,34]. Identified compounds will not only be important in screening of harvestings and selective breeding of plants for isolation and purification purposes, but they will also allow bioavailability analyses, and furthermore serve as quality control parameters of the final product.

This literature review will focus on the molecular mechanism of estrogen as traditional HRT source and how it translates to both the positive and the negative effects associated with estrogen supplementation. *Cyclopia* poses as a possible phytoestrogen source and as such phytoestrogen related topics, covering commercially available phytoestrogenic nutraceuticals and its popularity amongst menopausal women, as well as the rationale for the increased interest in *Cyclopia* as possible phytoestrogen source will be discussed. For the isolation and identification of active compounds from an extract, activity-guided fractionation is regarded as the most successfully employed technique, therefore, this literature review will provide background on this technique and its practise as well as discussing the various estrogenic tests available to test for activity. Furthermore, factors influencing absorption and metabolism of compounds will also be discussed as active compounds have to become bioavailable to elicit a therapeutic effect. The literature review will be concluded with a research rationale for the current study, from which the aims of the thesis will be formulated.

2.2 Estrogens

2.2.1 Sources of estrogen

Estrogens may be classified into two groups namely endogenous and exogenous estrogens. These two groups of estrogens not only differ in terms of source and estrogenic potency, but also in terms of the biological response they elicit.

2.2.1.1 Endogenous estrogens

Four different endogenous estrogens have been identified, with the three predominant estrogens being estrone (E_1), E_2 and estriol (E_3), and the less common estetrol (E_4). The three predominant estrogens are all steroids consisting of 18 carbon atoms and characterised by an aromatic A-ring [35]. The hydroxy groups at positions 3 and 17 and the aromatic A-ring are considered crucial for the specific estrogen effect [35] (Fig. 1B). The individual potencies of the endogenous estrogens vary, with E_3 the least potent and E_2 the most potent [36,37].

E_1, E_2 and E_3 are the main estrogens in non-pregnant women, and are synthesized by the aromatase enzyme (estrogen synthase) in the ovarian granulosa cells, various extraglandular sites like the skin and brain fibroplasts, and also, adipose tissue [38]. In the ovaries, the main site of estrogen biosynthesis in premenopausal women [39], the initial movement of cytosolic cholesterol into the mitochondrion is facilitated by the steroidogenic acute regulatory protein ($StAR_1$) (Fig. 1A). Six enzymes are then involved in the conversion of cholesterol to E_2 [40] (Fig. 1A&B). The aromatase enzyme, located in the endoplasmic reticulum of estrogen-producing cells, is responsible for estrogen formation from C_{19} androgens [41,42]. The enzyme is comprised of two polypeptiteds, namely, the flavoprotein, NADPH-cytochrome P450 reductase and the aromatase cytochrome P450 (encoded for by CYP 19) [38]. E_2 is converted to E_1 by the activity of 17β-hydroxysteroid dehydrogenase in a reversible reaction forming part of the E_2 biosynthesis pathway [35]. The principal product of the ovaries is the potent E_2, while the estrogenically weak E_1 is formed in large quantities in adipose tissue where at least half of it is converted to E_2 in extraovarian tissues [43].

In post-menopausal women E_2 levels are low as the ovaries cease to produce E_2. Consequently, the primary site for estrogen biosysnthesis is no longer the ovaries but the adipose tissue and the skin. In post-menopausal women the predominant estrogen is E_1, which originates from androstenedione [40,44]. The adrenal cortex is the primary source of androstenedione which serves as immediate precursors of E_1 [35]. E_1 serves as the primary source of E_2 synthesis in post-menopausal women, however, androstenedione can also be converted testosterone to form E_2. Even though adrostenedione is predominantly produced by the adrenal glands, some androstenedione is also produced by the ovaries. In addition, the adrenal gland and the ovaries also supply other androgenic precursors that are suitable for estrogen production [45,46].

During pregnancy, the predominant estrogen is E_3 which is formed from androgenic precursors provided by the mother and the fetus [35]. Androgenic precursors then undergo fetal liver hydroxylation, where after the placenta is responsible for the conversion of hydroxylated androgenic precursors to E_3 [47]. E_4 (Fig. 2A) is exclusively formed during human pregnancy [48], where fetal liver hydroxylation reactions are a necessity in E_4 synthesis from its precursors [49-51] (Fig. 2B). Produced E_4 then reaches the maternal circulation through the placenta [48]. E_4 synthesis can occur from phenolic E_2, E_3, 15α-hydroxyestradiol, or from neutral androgenic precursors (Fig. 2B) with sulfo-conjugation preceding hydroxylation where present [48,52-54].

Numerous biological roles of estrogens have been identified. During fetal life E_3 is responsible for cerebral development, leads to breast swelling and endorses uterine growth. E_2 promotes the advance of female sex characteristics, and also stimulates uterine growth and breast development. During menopause circulating estrogen levels decline and the estrogenically less potent E_1 becomes the predominant estrogen [35,56,57]. Consequently, this leads to atrophy of the genitalia and also many other menopause associated symptoms and diseases [35]. Uterine cell-differentiation, in contrast to the growth induced by E_2, has been identified as a biological role of E_4 [48].

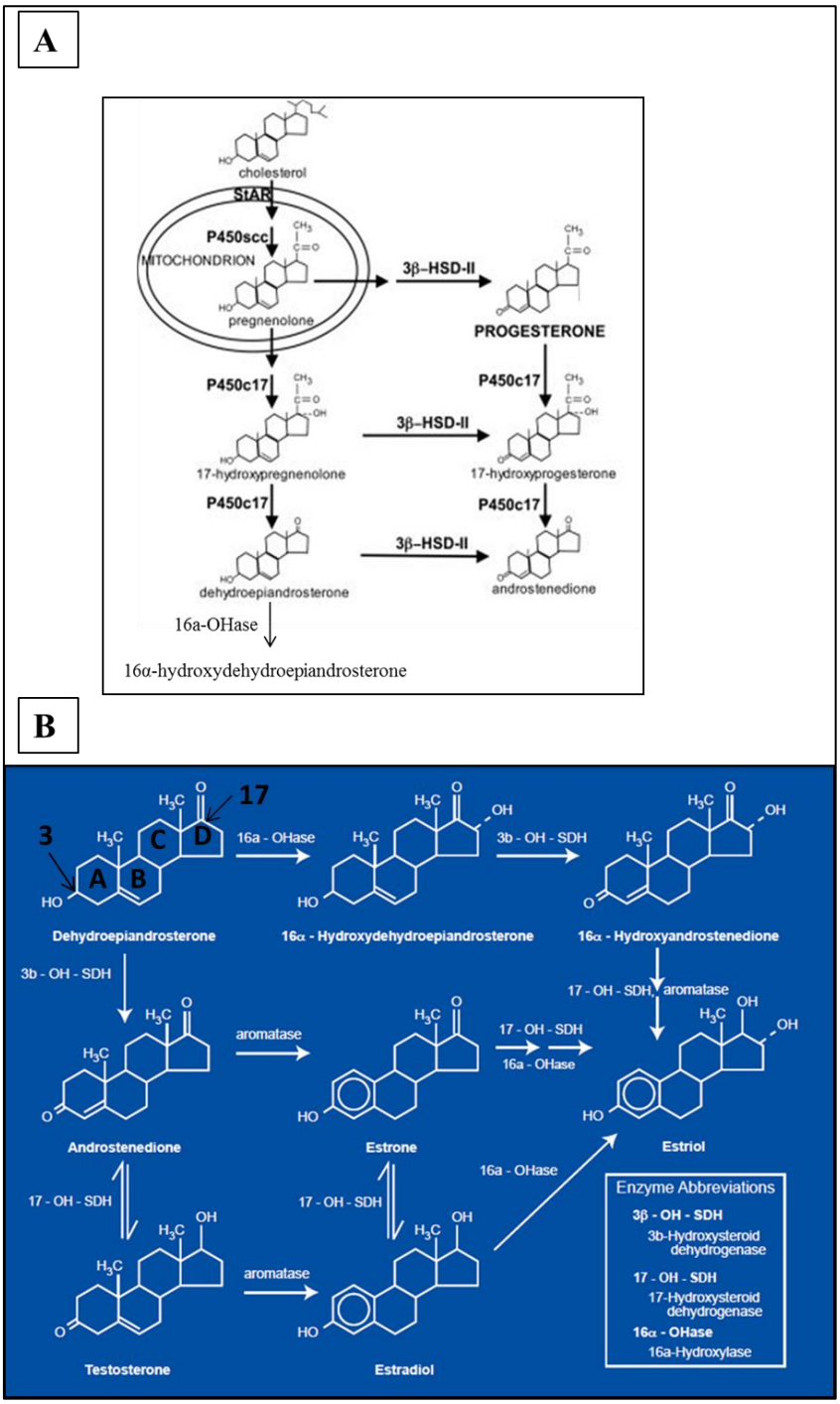

Figure 1. Biosynthesis of E_1-E_3 (Adapted from Wright *et al.* [55] and Bulun *et al.* [40]. (A) In the ovaries the StAR protein facilitates the main step of estrogen biosynthesis by enabling the entry of cholesterol into the mitochondrion which leads to progesterone and subsequent dehydroepiandrosterone. (B) Several enzymes are then responsible for the conversion of dehydroepiandrosterone to E_1-E_3.

Levels of endogenous estrogens can act as determining factors or markers for the detection of physiological abnormalities. E_2, with circulating levels between the pM to nM range in pre-menopausal women, is the physiological estrogen most frequently connected with the female reproductive phase function as well as the initiation and progression of cancers in the female

reproductive tissues [58]. E_1 ranges from 0.5-1 nM in reproductive and from 150 pM-200 pM in postmenopausal women, whilst E_3 is higher in pregnant, than in non-pregnant women (10-100 nM vs 7 nM). Low levels of E_3 in pregnancy have been correlated with the incidence of Down's syndrome in offspring [59] and complications of eclampsia [60]. E_4 levels in late pregnant females are approximately in the 3 nM range [48], and studies have shown that E_4 does not represent a unique marker for the identification of fetal abnormalities [48].

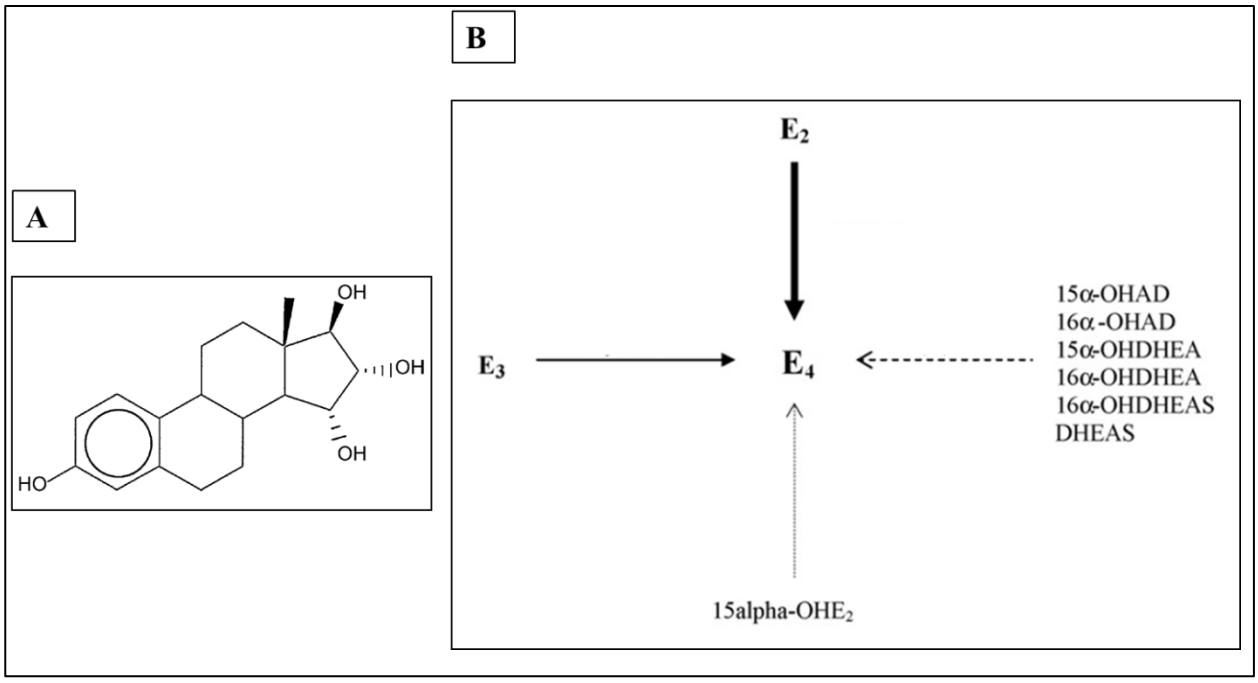

Figure 2. (A) E_4 chemical structure and (B) biosynthesis pathways as adapted from Holinka *et al.* [48]. Abbreviations used: E_3 = estriol; E_2 = 17β-estradiol; E_4 = estetrol; 15α-OHE_2 = 15α-hydroxyestradiol; 15α-OHAD = 15α-hydroxyandrostenedione; 16α-OHAD = 16α-hydroxyandrostenedione; 15α-OHDHEA = 15α-hydroxydehydroepiandrosterone; 16α-OHDHEA = 16α-hydroxydehydroepiandrosterone; 16α-OHDHEAS = 16α-hydroxydehydro- epiandrosterone sulfate; DHEAS= dehydroepiandrosterone sulfate.

In summary, four different endogenous estrogens, E_1-E_4 have been identified. E_1-E_3 are the only circulating estrogens in non-pregnant women, with E_2 as the predominant estrogen. During menopause the ovaries cease to produce E_2 which leads to a decline in circulating E_2-levels. Consequently E_1 is the predominant estrogen in post-menopausal women, and serves as the main source of E_2 synthesis. In pregnant women E_1-E_4 are all present, with E_3 as the predominant estrogen. Apart from the structural differences between E_1-E_4, they differ in their estrogenic potencies, and they have different biological roles. These endogenous estrogens, however, are not

solely responsible for all estrogen-induced biological responses, as exogenous dietary estrogens, upon absorption by the host, also play a role.

2.2.1.2 Exogenous estrogens

Dietary estrogens may be classified into two main groups, namely, naturally occurring estrogens and synthetic contaminant estrogens (Fig. 3) [61], where naturally occurring estrogens are divided into ovarian steroids, phytoestrogens and mycoestrogens, and synthetic contaminant estrogens are divided into pharmaceutical estrogens and xenoestrogens (Fig. 3) [61]. As ovarian steroids have already been discussed in section 2.2.1.1 this section will focus on phytoestrogens, mycoestrogens, pharmaceutical estrogens, and xenoestrogens. It is important to bear in mind that phytoestrogens form the largest part of dietary estrogens, and that they are also more common in the human diet, consequently they will be discussed in more detail.

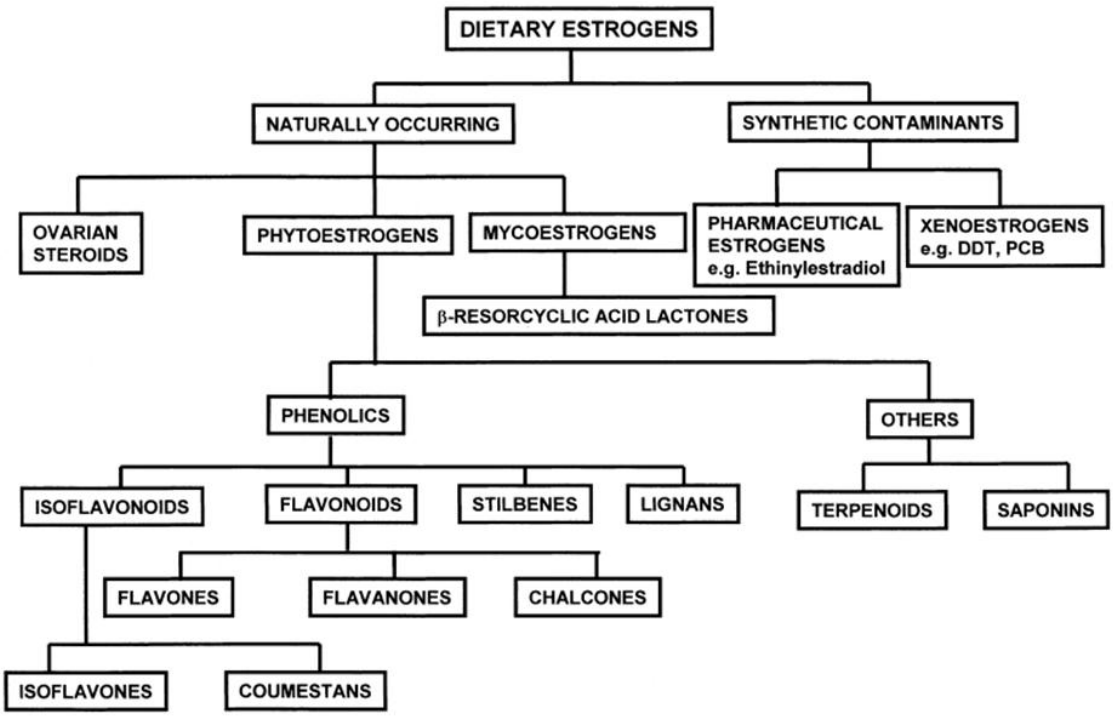

Figure 3. Assembly of dietetic estrogen groups [61]**.**

Phytoestrogens are plant-derived compounds that are structurally similar to mammalian E_2 and as such promote estrogenic actions in mammals [62,63]. They may be classified into isoflavones such

the aglycones genistein and daidzein or the glycones genistin and daidzin, coumestans such as coumestrol and 4'-methoxycoumestrol, flavones such as apigenin and luteolin, flavanones such as eriodicytol and naringenin [61], flavonols such as fisetin, catechins such as proanthocyanidins, lignans such as secoisolariciresinol and matairesinol and chalcones, such as phloretin. Although flavanols and catechins form part of the flavonoids this is not indicated in Fig. 3. In addition, stilbenes such as resveratrol [64] and the terpenoids and saponins (Fig. 3), such as tschimganidine and diosgenin [65], respectively, are also classified as phytoestrogens. The most studied sources of phytoestrogens include black clover, red cohosh, flax, licorice, hops and dong quai [65]. The main phytoestrogenic sources will be discussed in the next section of this literature review, where they will be assessed terms of their historical usage, and the benefits they may offer.

Even though mycoestrogens are metabolites of fungal species and not plant compounds, they are frequently discussed as part of the hierarchy and classification of phytoestrogens [61]. The naturally occurring mycoestrogen, zearalenone, is frequently found in food as a result of grain infection by *Fusarium* moulds [66]. Zeranol and zeranlenol are derived from zearalenone, they share weak estrogenic activity, and are collectively known as β-resorcylic acid lactones (RALs). Zeranlenol, a metabolite of zearalenone, is likely to be found in animal tissues, and it is known to compete with E_2 for ER binding and subsequent activation [67].

Synthetic contaminants also form part of dietary estrogens, and may be classified into two groups, namely pharmaceutical estrogens and xenoestrogens (Fig. 3). These estrogens will be briefly discussed in the following two paragraphs.

Xenoestrogens are considered synthetic non-steroidal small lipophilic compounds that copy the action of endogenous estrogens [68]. They originate from urban waste or consumer products, agricultural spraying, and industrial processes [69]. Xenoestrogens may be divided into two groups, namely, environmental pollutants such as fungicides and pesticides, which includes lindane, chlordecone, dichloro-diphenyl-trichloroethane (DDT) and atrazine, and the second group,

industrial chemicals such as surfactants and plasticizers, which includes polychlorinated biphenyls (PCBs), alkylphenols, bisphenol A and phthalate esters [70-73]

Pharmaceutical estrogens are also a class of synthetic estrogens, however, they were designed with an intended medicinal use. Ethinyl estradiol is an example of a pharmaceutical estrogen most commonly used in contraceptives such as Generess®. Another commercially available product containing ethinyl estradiol is Quinestrol®, which is used in hormone replacement therapy [74,75]. Selective estrogen receptor modulators (SERMS) such as tamoxifen [76], and selective estrogen receptor downregulators (SERDS) such as fulvestrant, both designed for the use in treatment of breast cancer [77], also form part of pharmaceutical estrogens, eventhough some phytoestrogens are also often referred to as natural SERMS [78].

In summary, exogenous estrogens may be divided into two main groups namely naturally occurring estrogens and synthetic contaminants, where phytoestrogens form the predominant group of naturally occurring estrogens, however, the estrogenic contribution of the other groups should not be discounted. Even though endogenous and exogenous estrogens differ in source, chemical structure and estrogenic potency, all estrogens mediate their effects through a similar signalling transduction pathway, and as such the next section will focus on the molecular mechanism of estrogen action.

2.2.2 Molecular mechanism of action

The signalling transduction pathway of all estrogenic compounds are similar to that of E_2, and as E_2 is the major endogenous estrogen in non-pregnant premenopausal women, the following section will in detail discuss the molecular mechanism of E_2 action.

E_2 action is mediated via the ER, which forms part of the superfamily of nuclear receptors functioning as ligand-activated transcription factors [79]. ERs are divided into two main subtypes, namely, ERα and ERβ (Fig. 4). Both contain two highly conserved regions, the E/F or ligand-binding domain (LBD), found at the carboxyl-terminal end, which binds estrogen, and also the C or

DNA-binding domain, found in the middle of the ER, which is involved with DNA interaction [79]. The LBD also has other functions such as nuclear localisation, dimerization, heat shock protein association, and hormone dependent activation function (AF-2). In contrast, the amino-terminal end (A/B domain) contains a ligand independent activation function (AF-1) [80]. ERα comprises a fully functional AF-1 and AF-2 domain, whereas ERβ lacks a functional AF-1 domain, but retains a functional AF-2 domain [81].

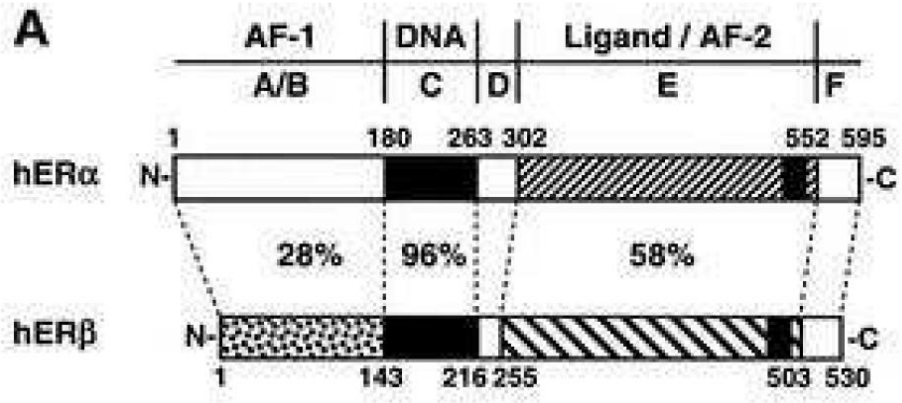

Figure 4. Diagram illustrating the % homology between human ERα (595 amino acids) and human ERβ (530 amino acids) [82]**.** DNA refers to the DNA-binding domain; Ligand refers to the ligand binding domain; AF-1 and AF-2 refers to the two transcriptional activation functions

Estrogen-dependent transcriptional activation (Fig. 5, point 1) occurs when E_2 diffuses across the plasma and nuclear membranes and consequently binds the ER. In the absence of E_2, the ER is in its inactive form bound to heatshock proteins within the nucleus [80]. Upon binding of E_2 to the ER, the ER transforms into its active state by facilitating the dissociation of the heatshock proteins and permitting dimerization of receptor proteins [83]. Following homodimerization, the ERs bind the ERE (estrogen response element), which is frequently situated in the 5'-flanking region of estrogen-responsive genes [80]. The DNA-bound receptor then either directly or indirectly interacts with the general transcription machinery via cofactors [84], such as PPARγ coactivator-1 (PGC-1), CREB binding protein (CBP)/p300, amplified in breast cancer-1 (AIB1), steroid receptor coactivator 1(SRC-1) and glutamate receptor-interacting protein 1(GRIP1), steroid receptor RNA activator (SRA), and p68 RNA helicase [85]. The receptor-cofactor-transcriptional machinery interaction

aids in the disturbance of chromatin at the ERE by stabilising the transcription preinitiation complex in order to exert either a positive or negative effect on the expression of target genes [81].

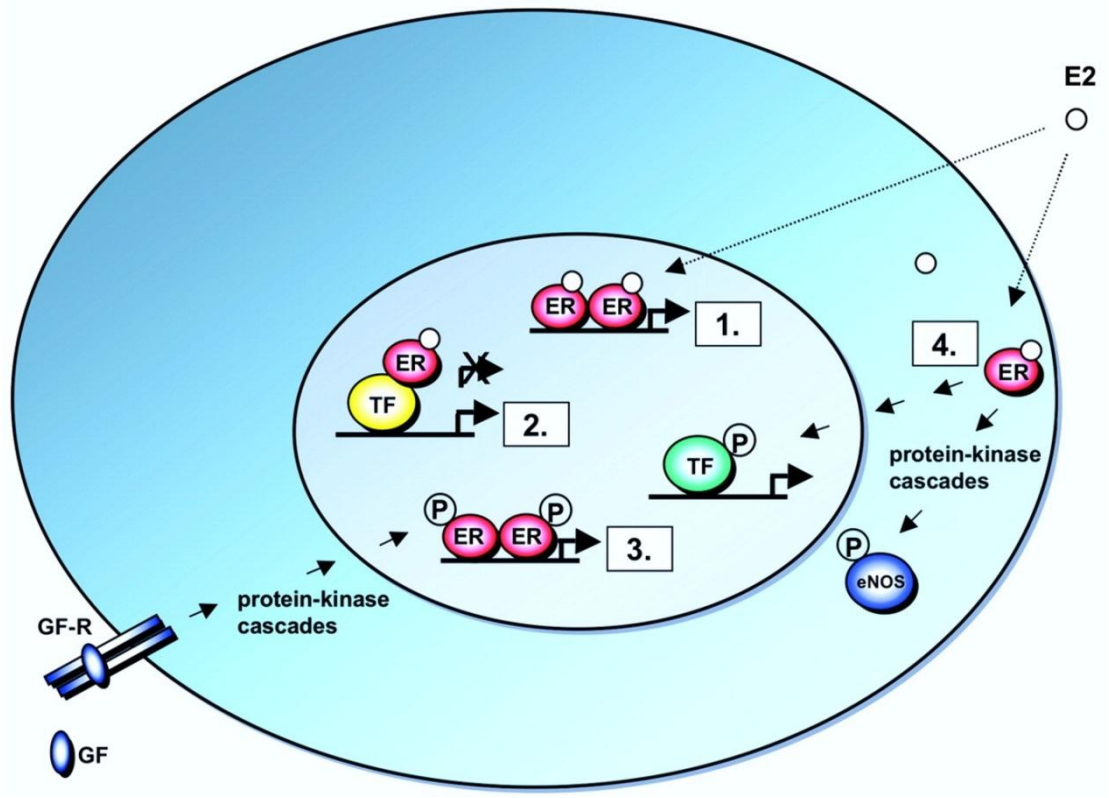

Figure 5. Schematic illustration of the ER mode of actions [86]**.** 1. classical ligand dependent actions, where E_2-induced ER in the nucleus binds to EREs in the promoter region of target genes, 2. ERE-independent actions, where nuclear E_2-ER complexes are bound to transcription factor complex (TF) that binds to the promoter region of target genes, 3. ligand independent actions, where nuclear ERs are activated via phosphorylation (P) by protein kinase cascades, initially activated by growth factors (GF), 4. non-genomic actions, where protein kinase cascades are activated by membrane E_2-ER complexes resulting in altered gene expression or protein function.

Activation or repression of genes can also occur in the absence of an ERE-site (Fig. 5, point 2) [81]. This mechanism is referred to as transcriptional cross talk or tethering [87] and covers E_2- induced activation or repression of several genes which commonly occur through binding of the ERs to other transcription factors. These E_2-ER-transcription factor complexes elicit an effect by binding to the promoter region of the target genes. Transcriptions factors include Fos and Jun proteins that bind AP-1 (activating protein-1) binding sites, ER-specificity protein-1 (Sp1), nuclear factor κB (NF-κB), CCAAT/enhancer binding protein β (C/EBPβ), or GATA binding protein 1 (GATA-1). Genes activated by E_2 through the interaction of ERs with Fos and Jun proteins at AP-1 binding sites include collagenase [88], ovalbumin [89], IGF-I [90] and cyclin D1 [91,92], however, E_2

activated ERs also repress certain genes such as the choline acetyltransferase gene [93] via ER-AP-1 complexes. ER-Sp-1 complexes are known to regulate genes that contain GC-rich promoter sequences [94] with genes such as c-fos [95], cyclin D1 [96], and low-density lipoprotein receptor (LDL-R) [97] known to be activated by E_2-induced ER-Sp-1 complexes. Interaction of the E_2-induced ERs with NF-kB and C/EBPβ represses the IL-6 gene [98,99], whilst the interaction of E_2-induced ERs with the GATA-1 transcription factor represses erythropoiesis [100]. Even though ERE-independent genomic actions do not involve DNA-ER receptor interactions, it does involve the DNA binding domain (DBD) of the receptors [88-91,93,99,101-103], and even though DNA binding does not occur, it is speculated that the DBD might be necessary for proper protein-protein interactions, or for the recruitment of additional co-regulator proteins to the promoter region [86].

ER function can also be modulated by extracellular signals in the absence of E_2 i.e. ligand-independent (Fig. 5 point 3) activation. It was proposed that ligand-independent ER actions might either serve as a mechanism to stimulate growth factor pathways and consequently amplify mitogenesis in ER-positive tissues, or alternatively, it serves as a mechanism of ER activation in the presence of low E_2 levels as found in males [81]. Ligand-independent activation and subsequent increase in expression of ER target genes predominantly takes place in the presence of insulin-like growth factor-1 (IGF-1), polypeptide growth factors such as epidermal growth factor (EGF) and also 8-bromo-cyclic adenosine monophosphate [104]. It has been shown that alteration of the phosphorylation state of the ER by cellular kinases is important in ligand-independent activation. For example, mitogen activated protein kinase (MAPK) phosphorylates the serine 118 residue of the human ERα AF-1 upon administration of IGF or EGF, resulting in activation of target gene transcription [105]. In addition, for ERβ, the MAPK encourages the recruitment of SRC-1 to the N-terminus, thereby stimulating activation of target gene transcription [106].

The non-genomic actions of E_2 (Fig. 5, point 4), which likely occurs through cell surface ERs that are linked to intracellular signal transduction proteins, was suggested due to the rapid biological

effects of E_2 observed in the breast, vasculature, bone and nervous system [81]. These actions include the activation of the MAPK signalling pathway, studied in bone [107,108], endothelial [109], breast cancer [110], and neuroblastoma [111] cells, and the phosphoinositol (PI) 3kinase signalling pathway in liver [112], breast cancer [113] and endothelial cells [109]. The non-genomic actions of E_2 also include the stimulation of cyclic adenosine monophosphate (cAMP) production [114,115] and the mobilisation of intracellular calcium [116]. Controversy exists on whether ERs responsible for non-genomic actions are classical or non-classical ERs, situated at the plasma membrane [116-118]. ERs have been found in the caveolae of cells, where endothelial nitric oxide synthase (eNOS) is activated through protein kinase-mediated phosphorylation [119,120]. It, however, seems that the LBD of plasma membrane ERα is sufficient for mediating non-genomic activities of E_2 [121], and that the activity is induced by the interaction of the ERs and various scaffold or signalling molecules [86]. Membrane ERs also activates the membrane tyrosine kinase receptors in various cell types, as in the case where E_2-induced ERα directly interacts with IGF-1, leading to an increase in MAPK signalling [122]. In addition, E_2-induced ERα stimulates the EGF receptor by a mechanism that involves activation of Src kinase, G proteins, and matrix metalloproteinases, thus activating protein kinase B (Akt) and MAPK activities [123].

In summary, E_2 mechanism of action may be divided into four categories, namely classical ligand dependent actions, ERE-independent actions, ligand independent actions, and non-genomic actions. In general estrogen-induced biological responses are tissue specific due to a differential ER-subtype expression.

2.2.2.1 ER subtype expression

Some homology exists between the two ER-subtypes (Fig. 4), however, both ERs have distinct functions in estrogen action. Tissue localisation of ER-subtypes is thus not only important in understanding the molecular mechanism of estrogen action, but it is also important in understanding the initiation and progression of various estrogen-related diseases.

Even though ERs are co-expressed in various tissue types such as the breast, vascular, ovary [124], and bone [125] (Table 1), ERα is predominantly expressed in the classical estrogen target tissues such as the cardiovascular systems, uterus [126], mammary gland and bone, whereas ERβ is mainly expressed in non-classical estrogen target tissues such as the urinary tract, prostate and ovary [124,127,128]. Interestingly, even though both ERs are expressed in the human brain [125], ERβ is predominantly expressed in the cerebral cortex and the basal forebrain [129,130], whilst ERα is predominantly expressed in the hippocampus region [131]. Similarly, in rats, both receptors are co-expressed in the brain with some regions indicating selective expression, however, in contrast to humans, ERα is predominantly expressed in the ventromedial hypothalamic nucleus and subfornical organ [132], whereas ERβ is predominantly expressed in the cortex and the hippocampus [132,133].

Table 1 Tissue specific expression of human ER subtypes [125]**.**

Organ/Tissue	**ER subtype**		**Organ/Tissue**	**ER subtype**	
	Erα	**ERβ**		**ERα**	**ERβ**
Heart	✓	✓	Adrenal	✓	-
Lung	-	✓	Kidney	✓	✓
Vascular	✓	✓	Prostate	-	✓
Bladder	-	✓	Testes	-	✓
Epididymus	-	✓	Brain	✓	✓
Pituitary	-	✓	Thymus	-	✓
Liver	✓	-	Breast	✓	✓
Muscle	-	-	Uterus	✓	✓
Fat	-	-	Endometrium	✓	✓
Gastointestinal tract	-	✓	Vagina	✓	-
Colon	-	✓	Fallopian tube	-	✓
Small intestine	-	✓	Ovary	✓	✓
Bone	✓	✓			

As for humans, ERα is predominantly expressed in the mouse hippocampus [134], indicating not only tissue specific, but also species-specific ER expression.

Reliant upon whether the ERs are co-expressed or expressed separately in a tissue type, homodimers (ERα/ERα or ERβ/ERβ) or heterodimers (ERα/ERβ) can be formed for activation of gene expression [135,136], with the ERα/ERα or ERα/ERβ dimers demonstrating a greater binding affinity to EREs [135]. Changes in the selective expression of the ERs have been observed in certain tumour types [125], for instance an increased ratio of ERα/ERβ mRNA has been observed in ER-positive breast carcinoma [137] and ovarian tumours compared to the normal tissue [138].

Due to the high degree of sequence divergence in the N-terminus between ERα and ERβ, together with the fact that studies have shown that the AF-2 domain functions independently in ERβ [139], the two ER subtypes functions differently [139,140]. This was confirmed in a study where ERβ was shown to interact with EREs in a ligand independent manner and also to attenuate the ligand-activated transcriptional activity of ERα [139]. Co-expression of ERα and ERβ *in vitro* showed preferential formation of hetero-dimers [135], implying a convergence between ERα and ERβ pathways. It has thus been proposed that in cells co-expressing ERα and ERβ, the overall estrogen responsiveness may be determined by the ERα:ERβ ratio [139].

The differential ER subtype function and expression has become increasingly important in the pharmaceutical industry as side effects of HRT in menopausal women is a significant health issue. The selective action of estrogens in different tissues may not only be explained by the distribution of ERs in the different tissues, but also by the differential binding affinities of the individual receptors [124]. Therefore, in light of the fact that ERα is known to be pro-proliferative and ERβ anti-proliferative, focus has shifted to the development of estrogens that are not only subtype specific, but also to incorporate natural alternatives such as phytoestrogens that have higher affinities for ERβ than ERα [141].

2.2.3 Estrogen related diseases

Estrogen-related diseases may be divided into two categories, namely diseases resulting from the presence of E_2 or diseases resulting from a deficiency in E_2. The next section will focus mainly on menopause as the primary E_2-deficient condition, and the use and outcomes of conventional therapies used for menopause. As the primary adverse outcome of conventional HRT is breast cancer [9,10], we will discuss the role of E_2 in breast cancer, and also breast cancer treatments currently in use, followed by a benefit and risk analysis of HRT in E_2 deficient associated diseases.

Menopause is defined as the point in the life of women when 12 successive months of amenorrhea is experienced, without an obvious intervening cause, such as surgical removal of the ovaries or dietary deficiencies [142,143]. The average age of natural menopause has been estimated as 50-51 years [144,145], where the cessation of menstrual cycles are ascribed to the loss of ovarian follicles resulting in an estrogen deficiency [146]. It is estimated that women are born with one to two million primordial follicles, and at age 40 only a few thousand remain [147], followed by a further depletion with time resulting in menopause [148].

Menopausal symptoms are experienced by 67 % of menopausal women and these symptoms are usually more severe after abrupt cessation of ovarian function as per oophorectomy [2]. Symptoms can be classified into climacteric and psychosomatic symptoms. Climacteric symptoms include night sweats, hot flushes, atrophic vaginitis and insomnia, whereas psychosomatic symptoms include emotional instability, poor concentration, and memory impairment [149]. Apart from the relatively short term symptoms of menopause, long-term and also more serious diseases include osteoporosis, CHD and Alzheimer's disease [2].

Conventional HRT to treat menopausal symptoms include estrogen for women without an intact uterus, or estrogen plus progestin for women with an intact uterus [150]. Common E_2 deficiency diseases include osteoporosis, obesity, cardiovascular disease and neurodegenerative diseases [2,151]. Although HRT alleviates or prevents some menopause associated diseases such as

osteoporosis, CHD and possibly Alzheimer's disease [152], HRT usage is accompanied by an array of increased risks including breast cancer, endometrial cancer, increased incidence of gall bladder disease [153,154], and an increased risk of venous thromboembolic events [155,156]. The best established benefit from HRT usage is the reduced risk of osteoporotic fractures, as the therapy results in increased bone density [157]. In contrast, the most adverse and important risk of HRT is the increased incidence of breast cancer, where animal studies have shown that exogenous estrogens can induce breast cancer [158].

E_2 exposure is associated with breast cancer development as risk factors associated with breast cancer mirror increased exposure of breast tissue to E_2 [159]. Currently there are two hypotheses postulated to describe the above mentioned relationship [160]. The first hypothesis states that binding of E_2 to the ER stimulates the proliferation of breast tissue. Increased cell division and DNA synthesis thus results in an increased risk of replication errors, which may involve mutations that result in abnormal functioning of cellular processes such as apoptosis and DNA repair [151]. In the second hypothesis the metabolism of E_2 results in the formation of by-products that are able to damage DNA, which again results in mutations [151].

Although 67 % of all breast cancers have ERα, mutations within ERα are uncommon, as it occurs in only roughly 1 % of primary tumours [161]. However, it has been suggested that the mutation frequency is higher in metastatic breast tumours, where some mutations have been correlated with estrogen independence and tamoxifen resistance [162,163]. Even though mutation frequencies are low in primary tumours and increases in metasteses, many single mutations have been shown to influence breast cancer cell behaviour [164].

It is estimated that roughly 7-10 % of the epithelial cells in normal breast tissue express ERα, the receptor correlated with proliferation [165], and it has been shown that the expression varies with the menstrual cycle [166-168]. In contrast to ERα expression, ERβ expression is considerably higher in normal breast tissue (80-85 %), it does not fluctuate during the menstrual cycle [169], and

is inversely correlated with proliferation of cells [170,171]. It has been shown that ER-positive breast cancers have a higher ERα:ERβ ratio, compared to normal breast tissue, and also that estrogen-independent breast cancers have low ERα:ERβ ratios [172]. Furthermore, high grade ductal carcinoma in situ, and invasive breast carcinomas contain reduced levels of ERβ [169,173,174]. This indicates that the ratio of ERα:ERβ may affect the course of tumorigenesis [164].

Various treatments have been used in clinical practice to kerb and eliminate breast cancer. These treatments are all premised on reducing levels or signalling by estrogens. Chemotherapy used in the treatment of breast cancer causes ovarian suppression [175,176], however, it has a toxic effect on the patient, resulting in a decreased quality of life [177]. In addition, resistance to chemotherapy is the main problem in the treatment of cancer patients [178]. Resistance can be either innate, which occurs prior to drug treatment, or it can be acquired, which occurs following drug exposure [178].

It has been shown that approximately one third of breast cancer patients respond well to endocrine ablation such as in the case of oophrectomy [179], however oophorectomy does not completely remove the source of endogenous estrogens [180]. Compounds named SERMs such as tamoxifen was initially used to reduce estrogen signalling [181]. Tamoxifen acts as an ER antagonist, thus inhibiting binding of E_2 to ERs [182] in breast tissue, and as such serves as a less invasive, but also more efficacious method than oophorectomy [182]. Tamoxifen, however, also has estrogen agonist activities in other tissues [183,184], which can result in, for example, an increased risk in uterine cancer [185] and thromboembolism [186]. Also, tamoxifen resistance can develop as a result of tamoxifen treatment [187]. Abovementioned factors led to the development of a SERD, fulvestrant, an ER antagonist also aimed at inhibiting the binding of E_2 to ERs, however, with no agonist activities [188]. Currently, aromatase inhibitors are the most effective treatments available in the treatment of estrogen-dependent breast cancer [40], as it has been shown to be better than

previously used ER antagonists [189,190] by blocking estrogen formation at its source of synthesis, resulting in a more targeted effect [40].

HRT-induced breast cancer is of great concern for women in menopause as it is considered the primary side effect associated with conventional HRT use. Thus the recent move toward using phytoestrogens instead of conventional HRT to treat menopausal symptoms has been spurred by the fact that more and more women perceive phytoestrogens as a 'natural' alternative [191,192] with less side-effects compared to conventional HRT [153-156].

2.3 Phytoestrogens

2.3.1 Phytoestrogens as an alternative to traditional HRT

In the next section phytoestrogens as a source of estrogen and possible attributes arising from its consumption will be discussed. We will also discuss why most phytoestrogens are considered important candidates for the development of an alternative HRT.

Epidemiological studies have shown that the incidence of breast cancer is lower in Asian than in Western populations and that this may be attributed to the consumption of soy isoflavones [61]. In addition, studies have shown that high soy consumption during childhood is correlated with a reduced risk of breast cancer [193-196]. Phytoestrogen consumption in general may also have a protective effect on some other E_2-related conditions such as osteoporosis [197], breast cancer [198], prostate cancer [199], cardiovascular disease [200] and menopausal symptoms [201], however there are some concerns regarding the possible hazards involved in high level consumption of phytoestrogens [202].

Phytoestrogens primarily bind both ERα and ERβ and transactivates via ERE, however, they generally bind ERβ with higher relative binding affinity than ERα [203,204]. For example, genistein binds ERβ with a 20 greater affinity than it binds ERα, resulting in a 30 times greater estrogenic potency for ERβ [204]. Phytoestrogens has a mechanism of action different from

classical estrogen and act similar to SERMs, which has tissue specific ER agonist and antagonist properties [205]. The biological importance of the potent ERβ activation by phytoestrogens is of clinical importance as it is known that a group of genes responsible for cell cycle suppression and the retardation of proliferation are up-regulated by ERβ activation [206]. For this effect to take place, however, cells must express ERβ.

Apart from primarily binding the ERs, phytoestrogens also have other mechanisms of action which could beneficial to the host. Genistein, the best characterised phytoestrogen [207] and the most active and abundant phytoestrogen in soy [208], inhibits protein tyrosine kinases (PTKs) in tissues such breast cancer and prostate cancer cells [209,210], which slows the growth rate of these cells. In addition, inhibition of PTKs in other tissues is responsible for the neuroprotectant [211] and cardiovascular improving effects of genistein [212]. Apart from the inhibition of PTK's, genistein can also inhibit DNA replication enzymes that are involved in carcinogenesis such as matrix metalloprotein, matrix metallopeptidase 9 (MMP9), and DNA topoisomerases I and II [213,214].

In summary, phytoestrogens are generally considered as a plausible source for the development of a nutraceutical that will serve as an alternative to conventional HRT due to the specific molecular mechanism of phytoestrogen action [205,206]. Literature available on phytoestrogen consumption-associated advantages also supports their use as an alternative to conventional HRT [215]. Consequently, a range of phytoestrogenic nutraceuticals are currently commercially available.

2.3.2 Phytoestrogenic nutraceuticals

Botanical sources of phytoestrogens include soy, black cohosh, red clover, flax, licorice, hops, don quai, kudzu root [65], evening primrose oil, ginseng, alfalfa, chasteberry, [216-219], and even coffee [220]. Soy has received the most attention in terms of research [221-223], followed by red clover (*Trifolium pratense*, Fabaceae) [219,224] and black cohosh (*Cimicifuga racemosa*, Ranunculaceae) [225,226]. In the following section these three main and most investigated sources will be discussed briefly [65].

The most abundant source of isoflavones in food is soybeans, a legume consumed worldwide, but most commonly by Asian countries. Soybeans are rich in genistein and daidzein [227,228]. Apart from the use of soy in the alleviation of menopausal symptoms [229], various studies have shown an association between the intake of soybeans and other health benefits, such as reduced risk of breast cancer, prostate cancer, cardiovascular disease, and atherosclerosis [198,230-237]. Investigation of the bioavailability of the two predominant isoflavones in soy, genistein and daidzein, revealed that initial hydrolysis of the sugar moiety, which is performed by intestinal β-glucosidases, is required for their uptake into the peripheral circulation [238]. In addition, it has been shown that isoflavone absorption occurs efficiently, but varies considerably among individuals [223,239,240]. Studies have, however, shown that the levels of the isoflavones in soybeans may vary up to 3 fold in the same cultivar from different geographical locations, and over time [228]. The variability in isoflavone levels is attributed to environmental conditions [228,241] such as variations in temperature, nutritional status or drought, light conditions or pest attacks [228]. A standardised commercially available soy extract originating from soybean, for the treatment of menopause associated symptoms is currently available under the tradename PhytoSoya®. Each capsule of PhytoSoya® contains 17.5 mg of isoflavones, consisting of 50 % daidzein, 30 % glycitein, 20 % genistein [242].

Red clover is a herb traditionally used by the Native Americans in the treatment of whooping cough, cancer and gout [243], and is also traditionally consumed in parts of Asia [244,245]. Red clover is also used in the treatment of menopausal symptoms as a result of its composition [229]. It contains genistein and daidzein and two other estrogenic compounds, formononetin and biochanin A [243]. Studies have shown contradictory data regarding the potential of red clover as cardioprotectant, as some studies showed no effects, whereas other studies showed an increase in HDL cholesterol and a reduction in tryglycerides [246-251]. Furthermore, Tice *et al.* [252] reported that red clover extracts were not effective in alleviating the amount of hot flushes experienced by menopausal women, concluding that, even though the standardised extract investigated in the study

displayed estrogenic activity *in vitro*, it did not translate to the relief of menopausal symptoms. Other studies have, however, showed a positive correlation with red clover consumption and an increase in bone mineral density [246,250] and formation of bone mineral markers [250]. In addition, it has been shown that red clover does not affect the endometrium, whereas soy-related isoflavones have been reported to have an effect in some studies [253,254], but not in other studies [255]. *In vitro* studies of soy and red clover indicate no effect on breast cancer cell proliferation [256,257]. Red clover-derived isoflavones are regarded as well absorbed in the gut as they are predominantly in the aglycone form [258]. An example of a standardized commercially available red clover extract supplement is Menoflavon®, which contains 64 % red clover extract with an average isoflavone content of 11 % [259].

Black cohosh grows in Eastern North America and belongs to the buttercup family (*Ranunculaceae*). Native Americans used the rhizomes and roots of the black cohosh plant for a variety of conditions including treatment of diarrhoea, stimulation of menstrual flow, childbirth, dymenorrhea and suppression of cough [225]. In a study four randomised, controlled trials investigating Remiferim®, which is a black cohosh extract containing triterpene glycosides such as 27-deoxyacetain [260], phenolic acids (isoferulic acid, fukinolic acid), flavonoids, volatile oils and tannins [225,261], in the treatment of menopausal symptoms [262], it was found that three of the four trails indicated that black cohosh relieved hot flashes. Black cohosh is not classified as a phytoestrogen [263], but its compounds act synergistically to augment an estrogenic effect [263]. The exact mechanism of action of black cohosh is not known, and as such no studies have been performed on the bioavailability of the extract [264]. Adverse effects associated with black cohosh extract consumption are deemed mild by some authors [265], whereas other authors have reported cases of induced muscle damage and cutaneous pseudolymphoma, respectively [266,267].

In general, women in menopause have shown great interest in commercially available phytoestrogenic nutraceuticals as an alternative to conventional HRT. These nutraceuticals have

mostly been effective in alleviating menopausal symptoms together with additional positive effects not seen with conventional HRT. In the next section we propose an exclusively South African source for the future development of a possible phytoestrogenic nutraceutical.

2.4 Honeybush (genus *Cyclopia*)

To date, various studies have demonstrated that *Cyclopia* may pose as a candidate source for the future development of a possible phytoestrogenic nutraceutical as extracts of some *Cyclopia* species displayed desirable phytoestrogenic attributes [24-26,268-272]. In the next section we will discuss the epidemiology, and the potential health benefits associated with *Cyclopia*. In addition, we will discuss previous studies performed on the evaluation of the estrogenicity of *Cyclopia* extracts. Furthermore, known polyphenols present in a candidate *Cyclopia* extract will be evaluated for the prospective biological activities they may induce.

The genus *Cyclopia*, which forms part of the leguminous Fabaceae family, is a member of the Podalyriea family [273]. The *Cyclopia* genus was discovered in 1808, and today, some studies ascribe 23 [274], whilst other studies approximate 24 species [275] to this genus. *Cyclopia*, also known as honeybush, is widespread in the fynbos biome (Fig. 6) and as such the plant grows in the mountainous regions and on the coastal plains of the Western and Eastern Cape Provinces of South Africa [27]. *Cyclopia* species have a low leaf to stem ratio and have adapted to frequently occurring fires in their natural habitats by either being sprouters or non-sprouters, where sprouters produce new coppice shoots after fires, and non-sprouters lack lignotuber, but are re-seeders [276]. The seeds only germinate upon scarification, brought upon by the fire [273].

Aerial parts of *Cyclopia* species, such as *C. subternata*, *C. genistoides*, *C. intermedia*, and *C. sessiliflora* have long been used in the manufacturing of a herbal tea [277-279], which is known to have a distinctive brown colour and a sweet honey-like aroma [269]. The sensory attributes of honyebush tea together with the fact that the tea is low in tannins and caffeine-free contributed to the popularity of this tea [269]. The potential health benefits from the consumption of polyphenol-

rich beverages such as tea dates back 5000 years, and evidence relating polyphenol consumption with health benefits are renowned [280-282]. Since polyphenols are heat-labile, and as such degrade during the 'fermentation' step in classical fermented tea, more recent studies have included research on the health benefits of unfermented (green) honeybush tea [269].

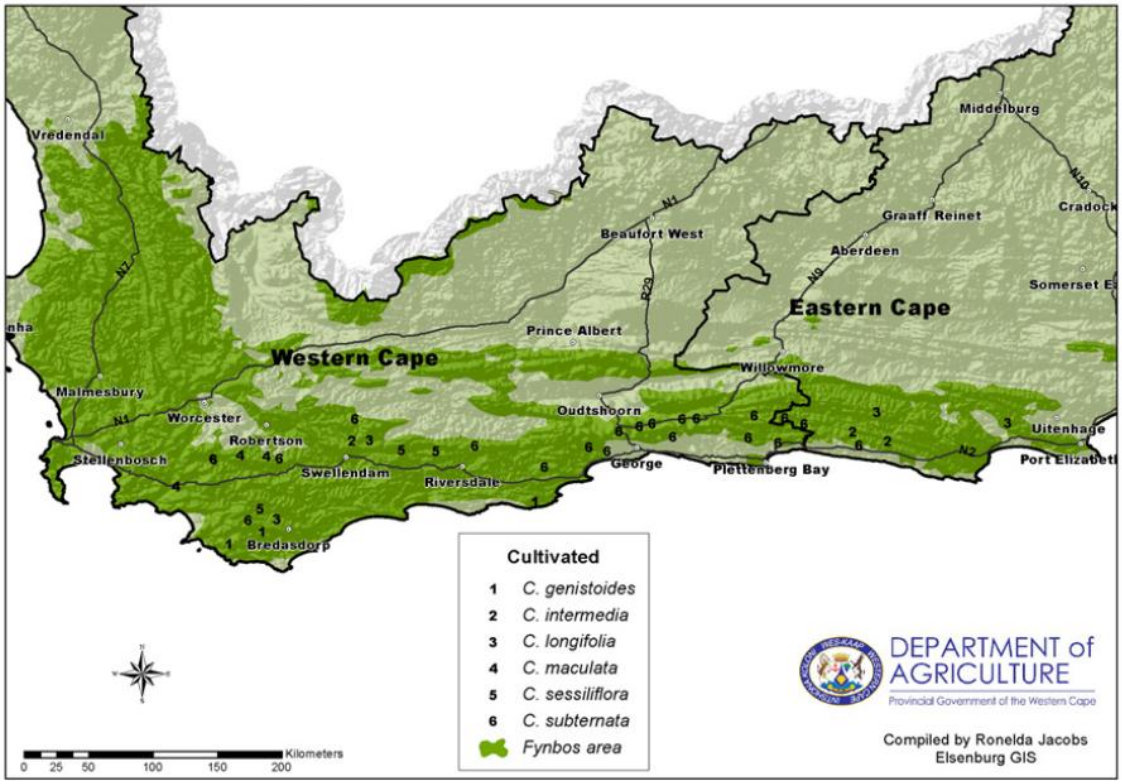

Figure 6 Natural distribution of *Cyclopia* species across the fynbos biome in South Africa [273].

Various *in vitro* and *in vivo* studies have validated the fact that *Cyclopia* has antimutagenic, antioxidant, anticancer and phytoestrogenic properties [269]. In the investigation of *Cyclopia* as a phytoestrogen source, *Cyclopia* extracts have been studied for estrogenic activity and its phenolic composition [24-26,268-272].

In a study by Verhoog *et al.* [24] in 2007, four different *Cyclopia* species *C. genistoides*, *C subternata*, *C. intermedia* and *C. sessiliflora*, were screened for phytoestrogenic activity, where *Cyclopia* extracts generally indicated a preferential binding to ERβ. ERα activation is associated with an increase in cellular proliferation, whereas ERβ activation inhibits ERα-mediated cell proliferation [165], thus it has been suggested that compounds, which antagonise ERα and/or

activate ERβ may be useful in the prevention and treatment of breast cancer [283-285]. Preferential binding to ERβ is thus a desirable trait in a phytoestrogenic nutraceutical, as it can allow alleviation of menopausal symptoms, whilst as a side-effect it may prevent breast cancer formation, the primary adverse outcome of conventional HRT [9,10]. Verhoog *et al.* [24] also demonstrated that methanol extracts from 'unfermented' (unoxidised) plant material, rather than aqueous extracts from fermented and/or unfermented plant material, generally displayed superior ER binding. *Cyclopia genistoides* and *C. subternata* extracts were examined for ER-binding, and it was found that unfermented extracts of both *C. genistoides* and *C. subternata* bound preferentially to ERβ. Also, binding of naringenin, formononetin, eriodictyol and luteolin, which are known phytoestrogens, and mangiferin, narirutin, eriocitrin (eriodictyol-7-*O*-rutinoside), hesperidin (hesperetin-7-*O*-rutinoside) and hesperetin, which were previously shown to be present in *Cyclopia* species [275,286], were screened for binding to ERα and ERβ. It was found that naringenin, formononetin and luteolin bound to both ERα and ERβ, however, preferentially to ERβ, with the order of radiolabeled E_2 displacement in a competitive binding assay being luteolin>naringenin>formononetin. Narirutin, eriocitrin and eriodictyol bound only to ERβ, whereas mangiferin, hesperidin and hesperetin did not bind to either receptor subtype. As the phytoestrogens, eriodictyol, luteolin, formononetin and naringenin, were not detected in any of the investigated extracts, accompanied by the fact that narirutin and eriocitrin, which binds ERβ, where present in only trace amounts (which could not fully convey extract estrogenic activity) led investigators to believe that further research might reveal novel compounds in *Cyclopia* mediating its estrogenic activity [24].

In another study by Verhoog *et al.* [25] DMEs from unfermented *C. genistoides* harvestings (P104, P105 and P122), and also polyphenols known to be present in *Cyclopia* species (luteolin, formononetin, mangiferin and naringenin), were evaluated for estrogenic activity [25]. P104 was the only extract that bound to the ER subtypes, however, unlike the phytoestrogens investigated, it indicated preferential binding of ERα. Nevertheless, P104 only transactivated an ERE containing

promoter reporter construct via ERβ, with a potency similar to the known phytoestrogen, genistein, and also E_2. In addition, although this extract induced breast cancer cell proliferation to a similar extent as genistein, but significantly ($p < 0.001$) lower than E_2, it could significantly antagonise E_2-induced proliferation of breast cancer cells [25].

In 2008 Mfenyana *et al.* [26] investigated four sequential and non-sequential extracts from different *Cyclopia* harvestings for phytoestrogenic activity. In the initial screening phase it was found that DMEs from M6 (*C. subternata*) and M7 (*C. genistoides*) plant material exhibited an appreciable degree of phytoestrogenic activity with M6 on average being quantitatively more efficacious than M7. In the next phase additional extractions of M6 and M7 were prepared and tested. Firstly, it was found that the M6-derived extracts, SM6EAc and NM6EAc, were the most efficacious of all other M6 and M7 extracts and also surpassed the efficacy of the original M6 and M7 DMEs. Secondly, it was found that two other M6-derived extracts, SM6Met and SM6Eth, were significantly more potent than all other M6 and M7 extracts, with SM6Met having the highest estrogenic potency of all extracts.

As SM6Met, a sequential methanol extract of a *C. subternata* harvesting, M6, showed the highest estrogenic potency of all the investigated extracts, it was investigated further in phase 3 of the study [26]. Phase 3 consisted of benchmarking SM6Met estrogenicity against commercially available phytoestrogenic nutraceuticals Phytopause Forte®, Femolene®, Remifemin®and Promensil®. Even though the efficacy of SM6Met was significantly lower than some of the commercial products in the promoter reporter and E-screen assays, the potency of SM6Met was not significantly different from Phytopause Forte or Femolene, while it was significantly higher than Promensil in the E-screen assay. In an attempt to identify compounds responsible for the estrogenic activity of SM6Met, selected extracts including SM6Met were analysed by means of HPLC and liquid-chromatography mass spectrometry (LC-MS). Narirutin, naringenin and formononetin, which have previously been shown to be present in some *Cyclopia* species [270], were not present in SM6Met,

however phytoestrogens such as eriocitrin and luteolin, were present. Even though eriocitrin were present in relative high quantities in the M6 extract, it appeared unlikely that eriocitrin was the sole cause of the estrogenic activity, as it could only partly displace ^{3}H-E_2 from ERβ, but not ERα [25], while the M6 dried methanol extract was able to fully displace ^{3}H-E_2 in MCF7-BUS cells. Luteolin also did not explain the estrogenic activity of the extract due to low quantities thereof in the SM6Met extract [26], thus the investigators suggested that other unidentified compounds possibly confer the observed estrogenic activity in SM6Met [26].

2.4.1 SM6Met and associated polyphenols

To date, two *Cyclopia* extracts have displayed sufficient estrogenic activity for future investigations. Firstly, P104, has been shown to bind to both ERα and ERβ, to transactivate only via ERβ in an ERE-containing promoter reporter assay, and to induce breast cancer cell proliferation, but at a significantly lower level than E_2, while antagonising E_2-induced breast cancer cell proliferation [25]. Secondly, SM6Met has been shown to have the highest estrogenic potency amongst other investigated *Cyclopia* extracts [26]. P104 plant material was, however, only available in small quantities, and as such SM6Met, which is available in bulk, seemed the most plausible candidate for future investigations. In the next section SM6Met estrogenic attributes will thus be discussed in further detail.

In vitro assays have shown that SM6Met transactivates via ERβ, and antagonises ERα activity in an ERE-containing promoter reporter assay [268], while it antagonises E_2-induced breast cancer cell proliferation [268]. In addition to the *in vitro* work, an immature rat uterotrophic assay was performed, which revealed that SM6Met, unlike E_2, did not increase uterine weight, and like the ER antagonist, ICI 182,780, antagonised E_2-induced uterine growth, which is indicative of ERα antagonism as seen in the *in vitro* assay [268]. The estrogenic traits of SM6Met, namely ERβ agonism, ERα antagonism and antagonism of E_2-induced breast cancer cell proliferation, are valuable traits needed in the development of a possible phytoestrogenic nutraceutical. An estrogenic

supplement with these traits will serve as a safer alternative to traditional hormone replacement therapy by not only activating anti-proliferative ERβ [287], but also by antagonising pro-proliferative ERα [288] and as such it should decrease the incidence of breast cancer, the primary adverse outcome of traditional HRT [289].

In a recent study LC-MS analysis of a *C. subternata* aqueous extract was performed [272], indicating the presence of 14 phenolic compounds (Fig. 7). In another study quantitative high performance liquid chromatography (qHPLC) analysis of SM6Met indicated the presence of protocatechuic acid and luteolin [268], and as HPLC is not as sensitive as LC-MS, some of the minor compounds previously detected in *C. subternata* [272] were not detected in this study. In addition, different extraction methods were used, which will affect the phenolic profile.

It is known that the xanthones, mangiferin and isomangiferin, are major constituents of *C. subternata* extracts. Even though isomangiferin has never been tested for estrogenic activity, mangiferin has been shown not to bind ERα or ERβ [24]. Interestingly, however, mangiferin has been shown to inhibit breast cancer cell proliferation via an ER independent mechanism [290]. In addition, mangiferin has been shown to have antioxidant [291], anti-inflammatory [292], enhancement of recall memory [293], anti-diabetic [294] and hypolipidemic activity [294].

Other polyphenols present in relatively high amounts in *C.subternata* extracts is the flavone, scolymoside (luteolin-7-*O*-rutinoside), and the flavanones, eriocitrin and hesperidin [268,270,272]. Eriocitrin, the glycone of eriodictyol, has been shown to bind ERβ, and as such serves as a phytoestrogenic compound [24], however, no research has been performed in elucidation of its estrogenic effect. In addition, eriodictyol has been shown to have weak estrogenic activity, and it has been shown to preferentially bind ERβ [295-297]. The 7-*O* rutinoside sugar moiety of eriocitrin may be hydrolysed by intestinal β-glucosidases prior to absorption [238,298], yielding eriodictyol. Eriocitrin and/or eriodictyol may thus partially contribute to the estrogenic activity of SM6Met, however, not as a whole, due to fact that they do not bind ERα, and as such they cannot confer the

ERα-mediated estrogenic effects of SM6Met [25]. Other properties attributed to eriocitrin include antioxidant and lipid-lowering activities [299]. Hesperidin has been shown not to bind the ER [24], or to transactivate an ERE-containing promoter reporter construct [300], and as such its presence cannot explain the estrogenic activity displayed by SM6Met.

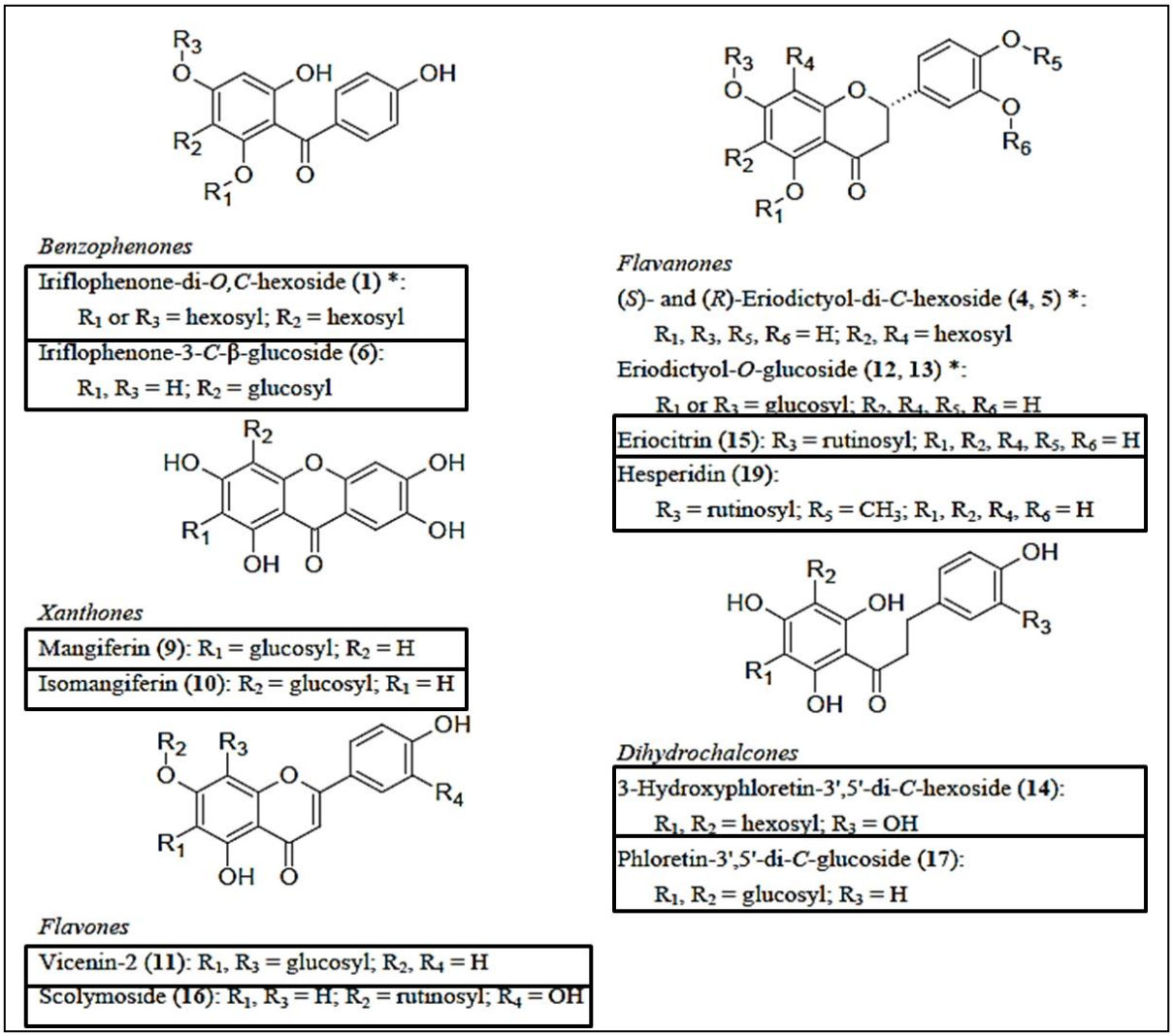

Figure 7 Chemical structures of phenolic compounds present in *C. subternata*. [272]. * indicates that the glycoside position is not yet known. Compounds previously identified in SM6Met as in Visser *et al*. [268] are indicated in text boxes. Compounds previously identified in SM6Met, not shown on the figure include the flavone, luteolin, and the hydroxybenzoic acid, protocatechuic acid.

Scolymoside, previously described as 'unknown 1' by Mfenyana *et al*. [26] is the product of a 7-*O* rutinoside sugar moiety addition to luteolin. Luteolin has been shown to bind both ER subtypes, induce breast cancer cell proliferation, and antagonise E_2-induced breast cancer cell proliferation [24,25,28,295,300,301]. Even though scolymoside has not previously been tested for estrogenic

activity [28,302], the 7-*O* rutinoside sugar moiety may be hydrolysed by intestinal β-glucosidases prior to absorption [238,298], yielding luteolin, and as a result increase the phytoestrogenicity of the investigated extract. Also, luteolin has exhibited anti-tumour activity [303], and as such luteolin and scolymoside may be regarded as chemo-preventative and as contributors, partially or as a whole, to the phytoestrogenic effects of interest displayed by SM6Met [268]. In addition, scolymoside has been shown to be an aldose reductase inhibitor [304], therefore it has strong radical scavenging properties, and its antioxidant activity was shown to be better than that of chlorogenic acid [305] and comparable to that of L-ascorbic acid [306]. Furthermore, scolymoside has also displayed antimicrobial activity [307].

Iriflophenone-3-*C*-glucoside belongs to the benzophenone polyphenolic class. Hydroxylated benzophenones such as iriflophenone, has been shown to display estrogenic activity in the human breast cancer cell line MCF 7 [308,309]. In addition, benzophenone derivatives can exert numerous biological effects including cytotoxic, antibacterial and antiviral effects [310]. Iriflophenone-3-*C*-glucoside also exerts *in vitro* antilipogenic activity [311]. It is thus plausible that this compound may exert synergistic effects with other *Cyclopia* constituents, i.e., mangiferin, hesperidin and (+)-pinitol, contributing to both cholesterol–lowering and anti-diabetic effects of honeybush observed in rat models [312].

Phloretin-3',5'-di-*C*-glucoside belongs to the dihydrochalcone compound class, and was previously described as unknown 2 [26]. Even though no research has been performed on its estrogenic activity, a previous study correlated estrogenic activity in SM6Met with the presence of phloretin-3',5'-di-*C*-glucoside [26]. Phloretin-3',5'-di-*C*-glucoside is a strong antioxidant and an important constituent of some citrus fruits like *Citrus microcarpa* [313] and *Fortunella japonica* [314], and as such it gives some insight into evaluated antioxidative properties of honeybush extracts [269,315].

In summary, the *C. subternata* sequential methanol extract, SM6Met, was identified as estrogenically the most potent amongst other *Cyclopia* extracts, and the potency was shown to be

comparable to commercially available phytoestrogenic nutraceuticlas [26]. Subsequent specific estrogenic activity evaluation of SM6Met indicated that it displayed estrogenic attributes desirable for the future development of a phytoestrogenic nutraceutical. These attributes included transactivation via ERβ, and antagonism of ERα activity [268]. In addition, SM6Met antagonised E_2-induced breast cancer cell proliferation [268]. The compounds previously identified in SM6Met, however, could not fully explain the estrogenic activity of SM6Met [26], which lead the researchers to believe that unidentified compounds, or compounds not previously tested for estrogenic activity are responsible for the specific estrogenic attributes of SM6Met. The most commonly used method for the identification of active compounds in a compound mixture or extract is activity-guided fractionation [33,34,316,317], which will briefly be discussed in the next section.

2.5 Activity-guided fractionation

Activity-guided fractionation is a technique that allows identification of active compounds from a mixture of compounds or from an extract. The procedure includes an assay or assays to determine the presence of the activity of interest. Central to this process are the fractionation steps, performed using a suitable fractionation technique. Lastly, activity-guided fractionation includes an identification step and possibly a quantification step [318]. In the next section the use of activity-guided fractionation will be discussed in general, where after the assays that can be implemented to track estrogenic activity will be discussed.

Activity-guided fractionation [317] is a technique that has long been the focus of many studies [33,34] for the isolation of compound(s) contributing to a specific activity of an extract. A recent example of activity-guided fractionation was done by Picot *et al.* [33] in 2011, where the compound responsible for the anti-proliferative activity in a *Dunaliella tertiolecta* extract was isolated. Here separation of the extract was firstly done by semi-preparative reverse phase high performance liquid chromatography (RP-HPLC). Each fraction was then tested in MCF-7 cells for anti-proliferative activity, where after the most anti-proliferative fraction was identified. This fraction was then sub-

fractioned using again the semi-preparative RP-HPLC technique and the sub-fractions were subsequently tested for anti-proliferative activity. RP-HPLC allowed collection of the most active sub-fraction. The absorption spectrum of the sub-fraction was then compared to a reference spectra and high resolution mass spectrometry confirmed that the active compound in the extract was violaxanthin.

Another example of a study utilizing activity-guided fractionation is a study performed by Hostettman *et al.* in 1999 [34], where compounds conferring antifungal activity to a whole plant *Parinari capensis* dichloromethane extract were isolated as follows: The extract was fractionated using HPLC, and peaks 2-4 where identified as major peaks using LC/ultraviolet (UV)/MS. Hereafter fractionation of the extract was performed using medium-pressure LC, silica gel column chromatography and Sephadex LH-20 gel filtration to produce constituents 2 to 4. Structure elucidation was done by 1D and 2D nuclear magnetic resonance (NMR), and it was revealed that compounds 2 and 4 were new compounds, where after the absolute configuration of compound 2 was determined. Evaluation of their anti-fungal activity revealed that constituents 2 and 3 inhibited *C. cucumerinum* growth.

In 2007 Cvoro *et al*. [319] identified the presence of ERβ-selective properties in MF101, an extract containing 22 herbs. In 2008, Mersereau *et al*. [316] identified the specific selective ERβ agonist compound, liquiritigenin, from the root of *Glycyrrhiza uralensis*, which is one of the plants found in MF101. The identification was made possible by initially fractionating the *G. uralensis* root-extract using gel chromatography. A promoter reporter assay identified the fractions with activity of interest, where after active fractions were purified further using preparative RP-LC. Using MS and NMR analysis, the identity of the compound was confirmed as liquiritigenin, a compound first isolated from *Glycyrrhiza* species [320].

The fractionation technique most commonly used in activity-guided fractionation is liquid chromatography (LC), which includes HPLC, low pressure LC, ultra performance (UP) LC, and

also CCC (counter-current chromatography). Other techniques also used include gel chromatography, thin layer chromatography, gas chromatography, and affinity enrichment. Advantages of the use of LC techniques include the ease to incorporate other liquid-based systems such as fractionators, autosamplers, and detectors, which simplifies the fractionation process, and makes the process more time efficient. Disadvantages include the use of organic solvents, not compatible with biological assays, which need to be evaporated and as such can result in the loss of volatile compounds.

CCC is a commonly used LC technique for the separation of compounds from a compound mixture or an extract [318]. More recently, two types of CCC have evolved from the original technique, namely high speed CCC (HSCCC) and high performance CCC (HPCCC). HSCCC differs from normal CCC by offering superior sample loading capacity, resolution, and shorter separation time [321]. In addition to the benefits of HSCCC, HPCCC works at a higher g-level and subsequently it has the ability to purify products more rapidly with a higher throughput [322].

Whereas conventional LC uses a single liquid phase in eluting analytes from the adsorptive or liquid phase coated solid support, the CCC technique uses a two-phase liquid solvent system, with one phase used as stationary and one as mobile phase [34]. This technique thus has an advantage over normal column chromatography through elimination of a solid support, as the danger of irreversible adsorption on the solid support is eliminated.

CCC instruments consist of a multilayer coil separation column conferring a type-J synchronous planetary motion, where the column holder rotates about its own axis and revolves around the centrifuge axis (Fig. 8) [321]. In an end-closed coil system (Fig 9A), planetary motion produces a hydrodynamic motion of the two immiscible solvent phases due to the Archimedean screw effect [321].

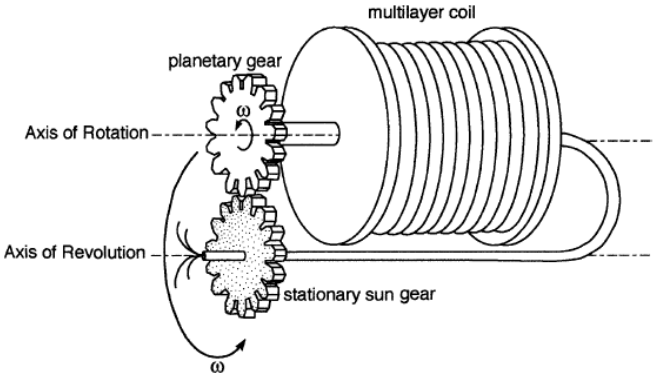

Figure 8 Schematic illustration of the type-J synchronous planetary motion of a multilayer coil separation column [321]**.** ω refers to the angular velocity.

Introduction of the heavier phase at the head will cause movement to the tail end, whereas introduction of the lighter phase at the tail end will cause movement toward the head, meaning that the mobile phase thus moves quickly through the coil leaving a large volume of the other phase stationary in the coil [321], allowing CCC separation as in Fig. 9B.

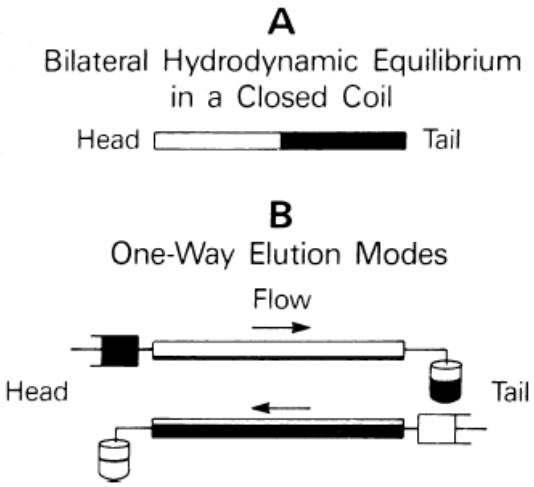

Figure 9 Mechanism of high speed counter-current chromatography (HSCCC) [321]**.** A) Bilateral hydrodynamic distribution of the two phases. B) Elution modes of both phases in the rotating coiled column

Drawbacks of the use of CCC as fractionation technique for activity-guided fractionation include the complexity of solvent system optimisation in order to obtain sufficient separation, and also the separation power of CCC has been reported as relatively limited [318]. Advantages of the technique include its robustness in allowing for a more time-efficient process [318], and it also allows no loss

of sample due to irreversible absorption as mentioned earlier [321]. In addition, no interfering surface chemistry, that can potentially alter analytes [323], is present in the use of CCC.

Detection methods commonly used in activity-guided fraction, which will not be discussed in detail, include the use of instrumental detection methods, such as MS and UV, and biodetector methods, such as segmented flow enzyme inhibition [318].

For the purpose of activity-guided fractionation of SM6Met the fractions obtained would have to be evaluated for their specific estrogenic activities. In the next section we will therefore discuss the various estrogenic assays that could possibly serve as estrogenic tests in activity-guided fractionation of SM6Met.

2.5.1 Tests to evaluate estrogenic activity

Various *in vitro* and *in vivo* assays are available to evaluate the estrogenic activity of a test substance. In 1998 the American Environmental protection agency proposed a two-tiered approach for the screening of endocrine disruptors including those affecting estrogen signalling. Tier I was aimed at detecting chemical substances capable of interacting with estrogen, androgen, or thyroid hormone systems through the use of a battery of suggested *in vitro* and *in vivo* assays. Tier II was aimed at determining the effects of compounds identified in Tier I on animals, and as such Tier II only consisted of suggested *in vivo* assays. Upon identification of the effects, Tier II was aimed at determining a quantitative relationship between the compound and its induced adverse effect [324].

Estrogenic tests for the evaluation of estrogenic attributes displayed by SM6Met and subsequent fractions should meet certain criteria. Firstly, the tests to be incorporated should be able to evaluate whether the attributes of interest are present or not. Therefore, the incorporation of a test that evaluates estrogenic activity via ERα or ERβ would be needed. In addition, a test to evaluate the effect of SM6Met and its sub-fractions on the proliferation of breast cancer cells would be needed. The second criteria that the tests should meet is that it should be easy to perform, require low

quantities of the test substance for evaluation, and yield as many results as possible, in a short period of time.

2.5.1.1 *In vitro* measurements for estrogenicity

Numerous *in vitro* assays to evaluate estrogenic activity are currently available. The most frequently used methods include binding assays, transactivation assays, and cell proliferation assays, which will be discussed in further detail in the next section. Due to variability of sensitivity of the various *in vitro* techniques available one should incorporate more than one of these techniques in evaluation of the estrogenic activity of a test substance. Other methods that will also be discussed in the next section include the analysis of E_2-induced mRNA and protein expression.

2.5.1.1.1 Receptor ligand binding assays

Various types of assays are available for measuring receptor-ligand binding interactions. In a review by Jong *et al.* [325] frequently used receptor-ligand binding assays are evaluated. Receptor-ligand binding assays can be categorised depending on whether a separation step is needed whereby bound ligand is separated from free ligand, or by means of the technique used for detection. Categorising assays according to the necessity of a separation step leads to three assay types namely heterogeneous, homogeneous and non-separating homogeneous [325] assays. Homogeneous assays require no washing or separation steps before measurements are taken, therefore it is known as mix-and-measure assay. An example of this assay is demonstrated in a study performed by Hu *et al.* [326], where a Time Resolved-Fluorescence Energy Transfer (TR-FRET) assay was used to investigate the ligand binding of a human G-protein coupled receptor (GPCR) protein. In non-separating homogeneous assays, described by Hemmila *et al*. [327], the signal is gathered from the target receptor immobilised on a solid phase, and as indicated by the type of assay, there is also no need for a separation. Heterogeneous assays require removal of the free from bound ligand by dialysis, filtration or centrifugation, resulting in an inconvenience for the experimenter. An example of a heterogeneous non-radioactive receptor assay is described in a study by Mahoney *et al*. [328] in

which the receptor-ligand binding is measured based on enzyme activity. Substrate was added to the enzyme-receptor-ligand mixture after unbound enzyme was washed away, where after absorbance measurements were taken.

The most frequently used technique for binding experiments is the use of a radioactive ligand for binding a receptor. This heterogeneous assay is based on competitive interaction between the non-labelled and labelled ligand for the receptor of interest. The appropriate radioactive ligand of choice must meet certain criteria to declare it fit for a study. These criteria are: the most potent enantiomer of the radioligand is ideal, the non-specific binding of the radioligand must be minimal, it must be chemically pure and have a high affinity for the receptor with a high specific activity, and it should be chemically stable and resistant to enzymatic digestion [329]. In a review written by Klinge *et al*. [330] it was stated that for a hormone binding to be a true receptor binding the following criteria must be met: the binding must be saturable (Fig. 10A), it must be stereospecific, tissue specific and hormone specific, and the binding must be reversible, and highly specific [330].

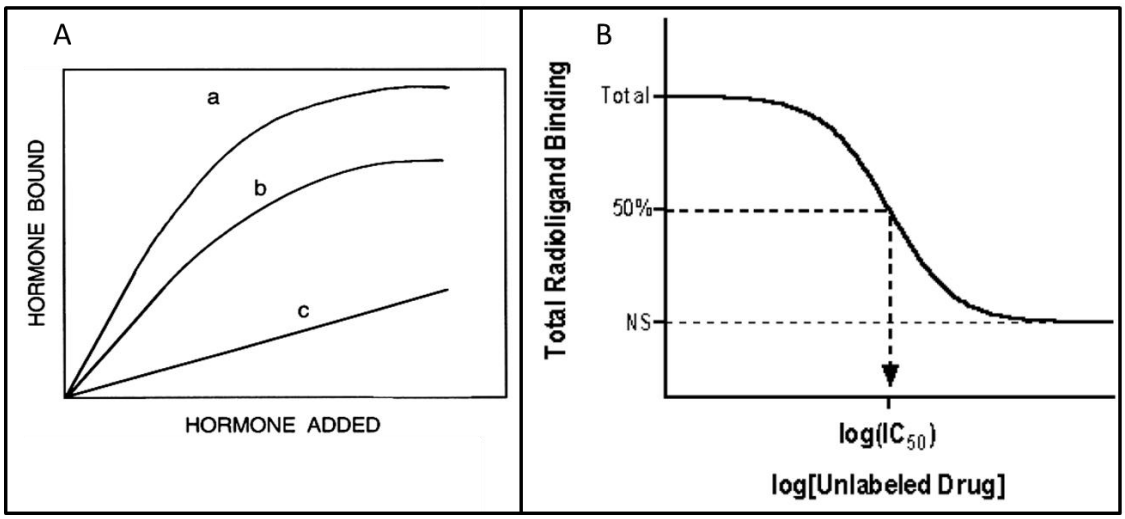

Figure 10 Graphical representation of (A) Saturation ligand binding, where (a) is total, (b) is specific and (c) is non-specific binding (b=a-c) [330] and (B) Competetive ligand binding [332].

Two common types of ligand binding assays may be conducted, namely saturation ligand binding (Fig. 10A) and competetive ligand binding (Fig. 10B). For saturation ligand binding increasing amounts of radioligand is added and B_{max} and the K_d are useful parameters that may be obtained.

B_{max} represents the total amount of receptors; while K_d is a measure of ligand binding affinity (Fig. 11). For competitve ligand binding increasing amounts of unlabelled ligand is added in the presence of one concentration of radiolabelled ligand. Useful parameters that may be obtained from competetive ligand binding assays include Y_{max} and IC_{50}. If the K_d of the radioligand is known, the B_{max} may be calculated from Y_{max} ($Y_{max}=B_{max}.[L]/(K_d+[L])$) and the K_i (the affinity for the unlabelled competing ligand) may be calculated from the IC_{50} ($K_i=IC_{50}/(1+[L]/K_d)$). In a study by Gutendorf *et al*. [331], evaluating competitive binding of E_2 and genistein to human ERα and ERβ, it was found that the IC_{50} values of genistein for ERα and ERβ was 35 µM and 2 µM, respectively, whereas for E_2 it was 3.5 nM and 65 nM, respectively.

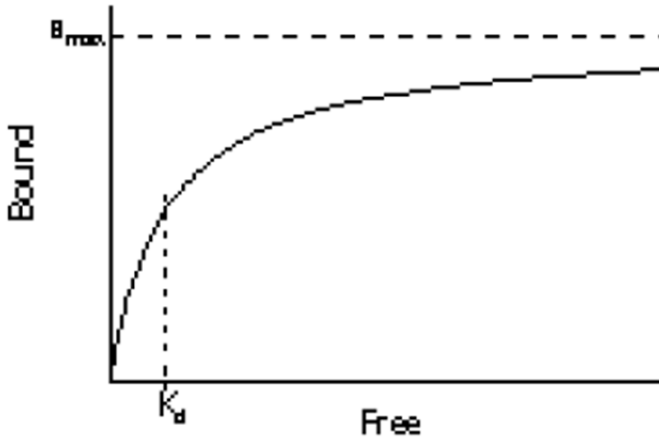

Figure 11 Graphical representation of bound ligand (Y-axis) plotted against free ligand (X-axis) [332]. The B_{max} value represents the total amount of binding sites and K_d the amount of ligand needed to occupy 50% of the total amount of binding sites.

Advantages of the use of radioactive ligands to investigate receptor-ligand interactions include the use of a single labelling step and the assay's specificity and sensitivity [325]. Binding techniques can also serve as a screening tool, meaning that if extracts or fractions do not bind the ER, further investigation in other assays will not be needed as estrogenic attributes will not be present. Disadvantages includes the inconvenience caused be the separation step and the use of radioactivity, which can be harmful to the experimenter. In addition, for the purpose of evaluating estrogenic activity in activity-guided fractionation, binding techniques will only demonstrate binding to the ER, and as such it will not give insight into the specific biocharacter of the estrogenic activity of the

extract or fraction investigated. Examples of commonly used *in vitro* estrogenic assays that do, however, give insight into the specific biocharacter of the estrogenic activity of a compound of interest, is the promoter reporter gene assay, the breast cancer cell proliferation assay, and analysis of mRNA and protein expression.

2.5.1.1.2 Promoter reporter gene assays

In the classical estrogen signalling pathway binding of a ligand to intracellular ERs results in ER-dimerisation, followed by the binding of these ER-dimers to an ERE present in the promoter region of estrogen responsive genes [333]. To study these events, the promoter reporter gene assay measures the estrogenic activity in an indirect manner based on the expression of an ERE linked reporter gene, which is under promotional control of the ERE (Fig. 12) [334]. In this assay a mammalian cell line or a yeast strain can be transfected with the ERE-containing promoter reporter construct, together with the ER cDNA if the cell line does not express the ER endogenously. Subsequently, upon ER activation the expression of the reporter gene is measured and quantified.

The promoter reporter assay can thus be used to determine the potency, or EC_{50} values of compounds, which can be compared to the EC_{50} value of E_2 [334]. The potency refers to the concentration of a ligand needed to induce a response halfway between the baseline and the maximal response. The baseline and maximal response is obtained by constructing ligand dose response curves. Similarly, the efficacy values of a compound can be determined, and compared to the efficacy value of E_2. Efficacy refers to the maximal response induced by a ligand.

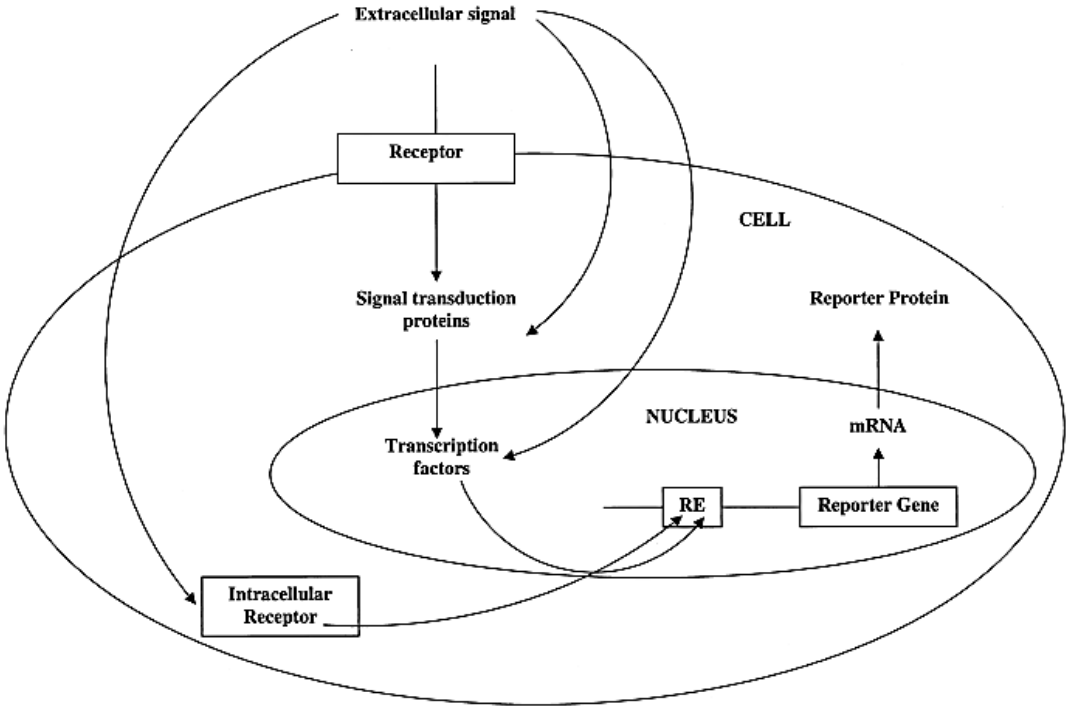

Figure 12 Graphic depiction of cell-based reporter gene assay [335]. Extracellular signals causes binding of either transcription factors or intracellular receptors to response elements (RE), resulting in the transcription on expression of the reporter protein.

Commonly used reporter genes include chloramphenicol acetyl transferase (CAT), B-galactosidase, luciferase, alkaline phosphatase, and green fluoresescent protein (Table 2). From Table 2 the most advantageous reporter gene to include in a promoter reporter assay for activity-guided fractionation of an extract would be the firefly luciferase reporter gene due to the numerous advantages of firefly luciferase reporter gene usage, such as amongst others, its specificity and broad dynamic range, accompanied by no significant disadvantages, as the substrate of this assay is commercially available.

Luciferase originates from a family of enzymes that are responsible for the oxidation of substrates such as luciferin, which consequently results in light discharge [335]. Three types of luciferase reporter genes currently in common use are the firefly (*Photinus pyralis*) luciferases, the bacterial luciferases, and in more recent times, the Renilla luciferase (*Renilla reniformis*) [336,337].

Table 2. Evaluation of frequently used reporter genes [335]**.**

Reporter gene	Advantages	Disadvantages
Chloramphenicol acetyltransferase (CAT) (bacterial)	1. No endogenous activity 2. Automated ELISA available	1.Narrow linear range 2. Use of radioisotopes
β-galactosidase (bacterial)	1. Well characterised 2. Stable 3. Simple colorimetric readouts 4. Sensitive bio-or chemiluminescent assays available	1. Endogenous activity (mammalian cells)
Luciferase (firefly)	1. High specific activity 2. No endogenous activity 3. Broad dynamic range 4. Convenient assays	2. Requires substrate (luciferin) and presence of O_2 and ATP
Luciferase (bacterial)	1. Good for measuring/analysing prokaryotic gene transcription	1. Less sensitive than firefly; not suitable for mammalian cells.
Alkaline phosphatase (human placental)	1. Secreted protein 2. Inexpensice colorimetric and highly sensitive luminescent assays available	1. Mostly endogenous activity 2. Interference with compounds being screened
Green fluorescent protein (GFP) (jellyfish)	1. Autofluorescent (no substrate needed) 2. No endogenous activity 3. Mutants with altered spectral qualities available	1. Requires post-translational modification 2. Low sensitivity (no signal amplification)

ELISA refers to enzyme-linked immunosorbent assay

Renilla luciferase [26] can catalyse the oxidation of membrane permeable coelenterazine, thus allowing quantification of gene expression of intact systems (i.e. a living cell). Bacterial luciferases are dimeric, heat-labile proteins, which limit their use, however, a rather sensitive assay has been developed using bacterial luciferases [338]. The most commonly used luciferase gene is the firefly luciferase, which produces 62 kDa protein requiring no post-translational modification for enzymatic activity [339,340]. The firefly luciferase reporter gene is commonly used is due to fact that it responsible for a reaction that is highly efficient [336], with a quantum yield of

bioluminescence < 0.88 [341]. Additionally, the firefly luciferase reporter gene is highly sensitive and has an expansive linear range of roughly up to 7 to 8 orders of magnitude [342,343], with little to no background noise in host cells. Firefly luciferase is vulnerable to proteolysis and as such it is fairly unstable in mammalian cells [344]. Its stability in lysis extract at 4°C is 6 weeks [345,346], whereas it is stable -80°C [336].

Estrogenic test substances are regarded as either agonists or antagonists, depending on their behaviour compared to known standard ER agonists such as E_2, or ER antagonists such as ICI 182 780 [347]. Antagonists, in contrast to agonists, are defined by their inability to elicit a response in a cell or tissue system on their own, while opposing standard agonist-induced activity.

In the proper use of any assay, positve and negative controls need to be in place to guard against false positive and negative results. Therefore in the evaluation of the estrogenicity of a test substance where either ERα/ERβ is co-transfected with the promoter reporter construct, the cells that undergo transfection should not express ER endogenously. The amount of the repective DNA constructs transfected, and the total amount of DNA transfected should also be constant. In addition, to control for the fact that the test substance solvent (vehicle) does not have an effect on the estrogenic result, the vehicle has to be evaluated on its own (in quantitative accordance as in a normal treatment condition) and compared to cells not treated with the vehicle. Inclusion of a standard positive control, such as E_2, will prevent false negative results.

An example of a promoter reporter assay for evaluation of estrogenic activity, was performed by Duong *et al*. [348] in which HeLa cells were transfected with an ERE2-TK-LUC promoter reporter constuct and either CMV-ERα or CMV-ERβ. Treatment with increasing concentrations of E_2 revealed an EC_{50} value of approximately 10^{-11}M for ERα, whereas it was approximately $10^{-10.5}$M E_2 for ERβ.

2.5.1.1.3 Cell proliferation assays

Various methods to measure cellular proliferation exist. These methods are aimed at either quantifying the ability of cells to metabolise dyes, to determine the protein content of cells, counting cells with a haemocytometer or cell counter, or measuring radiolabelled thymidine incorporation. In the next section the pros and cons of most of these assays will be discussed, however, as the use of metabolising dyes are most common in determining cell proliferation [349] it will be discussed in further detail.

Cell counting as measurement of cell proliferation using the traditional hemocytometer is usually very accurate, but this procedure cannot be used in high-throughput screening as the assay is laborious and time-consuming [350]. Another very accurate method involves the detection of tritiated thymidine fusion into the DNA of the cells [351], however, this method is also laborious, cost-ineffective, and requires safety measures due to the use of radioactivity [352].

Given these constraints, this resulted in the development of an array of other methods, which allows measurement of metabolic activity as an indicator of cell viability, and as such, cell proliferation. These assays include measurement of reduction of tetrazolium salts [353,354] and resazurin [355] via the action of mitochondrial enzymes, and measurement of cell esterase activity [356], glyceraldehyde-3-phosphate activity (G3P) [357], and cytosolic acid phosphatase activity [358]. These assays have proven to be very sensitive [359,360], however, as they measure cellular activity, which varies substantially over time, numerous measurements need to be made at specific time intervals for a substantial period of time, which can make the assays inconvenient [352]. In addition, as cellular nucleic acid is a sensible pointer of cell number [361], fluorescent indicators of DNA, such as 4'6-diamidino-2-phenylindole-2HCl (DAPI) [362], propidium iodide [363], and Hoechst 33258 [364], have made cell quantitation assays faster, but, roughly 10 times less sensitive than that of the newly developed methods described above [352].

Assays involving the use of tetrazolium salts include the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide) assay, the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), and the WST (water soluble tetrazolium) assay. In the MTT assay, the most frequently used cell proliferation technique to screen for proliferation of honeybush extract-induced breast cancer cells [25,28,268], tetrazolium salts are reduced by mitochondrial and extra-mitochondrial dehydrogenases to form insoluble blue formazan crystals, and as such an extra solubilisation step is needed before absorbance values are obtained [365]. Disadvantages of the MTT assay is that damaged mitochondria may still be able to reduce MTT to formazan, and that the substrate is toxic to cells [366-369]. In addition, the MTT assay has been shown to underestimate the anti-proliferative activity of certain polyphenols [370]. Advantages, however, include the fact that the colorimetric reaction occurs relatively fast [367], and the assay is non-hazardous, accurate and reproducible [371]. In the XTT assay the substrates are analogous to MTT, however, the formazan product is soluble, and another advantage of this assay is that the reactions are carried out intracellularly in the presence of phenazine methosulfate (PMS), which enhances the assay's sensitivity. In contrast, a disadvantage of this assay is that fatty acids, serum albumin and phenol red in cell culture media have all been shown to impair data collected from XTT and WST assays [372]. In WST assays the substrate is cell-impermeable and is reduced extracellularly by plasma membrane electron transport to generate soluble formazan [373] in the presence of PMS. Disadvantages, as with the other tetrazolium salt based assays, is the fact that cell metabolic processes vary under different cell culture conditions, and that these assays measure cell proliferation indirectly [374]. Advantages of WST assays, however, include the fact that it is more cost efficient than the MTT and XTT assay, and also, the formed product is water soluble, which allows for a more time efficient assay.

Analysis of dose response curves constructed from results obtained from an MTT assay, as in the case of the promoter reporter gene assay, allows for the determination of ligand potency and efficacy. An example of a study used to measure cell proliferation of breast cancer cells was

performed by Van Meeuwen *et al.* [375] in 2007. They incorporated the use of an MTT assay to evaluate the proliferation of MCF-7BUS cells after E_2 and genistein treatment and consequently an EC_{50} value of 38 nM and 5 pM was obtained for genistein and E_2, repectively.

2.5.1.1.4 Analysis of estrogen-induced mRNA and protein expression

The effect of a test substance on protein level is mostly evaluated by means of Western Blot analysis, whereby quantification of a specific protein that serves as a marker for estrogenicity is evaluated. Proteins that have been identified as markers for estrogenicity include the progesterone receptor (PR), present in breast tissue and the uterus [376,377], position specific antigen 2 (pS2), present in breast cancer cells [378], and cathepsin D, expressed in MCF-7 breast cancer cells [379]. In addition to measuring protein levels, quantification of estrogen responsive enzymes is also a possibility, which can be done using an alkaline phosphatase assay to measure activity of this enzyme [26].

The evaluation of the estrogenicity of a test substance can also be determined at the RNA level, where the mRNA encoding for the abovementioned marker proteins is investigated. These techniques include mRNA quantification using Northern Blot analysis, quantitative polymerase chain reaction (QPCR), and DNA microarray techniques [380,381]. The DNA microarray analysis of gene expression encompasses one major advantage, which is the fact that it allows the investigator to monitor the regulation of a variety of genes of interest. In a study by Coser *et al.* [382] a large number of estrogen regulated genes were investigated after exposure to a range of E_2 concentrations. This allowed investigators to categorise these genes into six categories, namely, very high E_2 sensitivity, high E_2 sensitivity, moderate E_2 sensitivity, low E_2 sensitivity and E_2 suppressible genes.

As abovementioned *in vitro* techniques, compared to *in vivo* techniques, are usually cost-effective, not time-consuming, and allows the investigator to do several assays in a relatively short period of time, it is important to note that the use of *in vitro* techniques to determine for instance

phytoestrogen estrogenicity, is also coupled to some limitations. These include the fact that *in vitro* assays can be inconsistent due to differences in cell line strains, media compositions, and serum sources [383,384]. Most importantly, the ability of *in vitro* assays to accurately mimic the responses of *in vivo* assays is questionable due to the fact that *in vitro* assays do not possess the same metabolic competencies and as such can produce false positive results due to, for example, its incapability to metabolically inactivate an estrogenic substance [385,386].

2.5.1.2 *In vivo* measurements for estrogenicity

In an attempt to comprehensively evaluate a substance, such as a possible phytoestrogen containing extract, for estrogenic activity, it is important to link the results obtained from as many as possible *in vitro* estrogenic assays with those obtained from as many as possible *in vivo* estrogenic assays. Two commonly used *in vivo* assays for assessing estrogenic activity include the uterotrophic and vaginal cell cornification assays [387-391].

For the uterotrophic assay, increased uterine wet weight relative to rat body weight is the most commonly used *in vivo* marker to establish estrogenicity of a test substance [389,392]. For the proper use of this assay standardized operating procedures need to be employed, as variables such as different species may result in different study outcomes [383]. It has, for example, been shown that the mouse uterus is more responsive to estrogens than the rat uterus [389]. Other variables to be considered is the fact that ovariectomised animals display greater sensitivity in the uterotrophic assay [393,394], and that the uterus is not only responsive to E_2, but also to compounds such as testosterone and progesterone related compounds [395-399]. This can influence results when investigating extracts for estrogenic activity, as extracts represent complex mixtures, of which many constituents are not characterised in terms of biological activity. As the uterotrophic assay only measures uterine weight, inclusion of other endpoints, such as the vaginal opening, cardiovascular system, liver and bone tissue, the mammary gland, and the brain, can also explain test substance organ or tissue specificity [400].

The vaginal epithelial cell cornification assay is believed to be a conclusive *in vivo* test regarding the estrogenic activity of a test substance [401]. The assay investigates the percentage of cornified cells in a vaginal smear sample collected from a treatment group of animals, relative to the percentage of cornified cells in a vaginal smear sample collected from animals in a control group. As an increased vaginal epithelial cell cornification percentage is indicative of test substance estrogenicity [402], the animal group treated with estrogenic compounds will display a higher percentage of vaginal epithelial cell cornification. Advantages of this assay include the fact that the assay is relatively easy to perform, and the same animal can be used for multiple tests as long as the test substance does not bioaccumulate. In contrast, a main disadvantage of this assay is that it is rendered time-consuming as the treatment period usually lasts for roughly three weeks, and a large number of animals need to be evaluated in order to acquire accurate results [389,391].

In conclusion, it is thus apparent that the use of single assay type is not sufficient for evaluation of the estrogenicity of a test substance, but that the investigator should rather employ a series of both *in vitro* and *in vivo* assays in an attempt to accurately predict the estrogenic potential of a test substance such as a possible phytoestrogenic source. It is important, however, to remember that *in vivo* assays are time-consuming assays, and as such, for the purpose of efficiently and accurately performing activity-guided fractionation of an extract, the incorporation of more than one *in vitro* assay would be needed.

2.6 Absorption and metabolism

As mentioned earlier SM6Met serves as a candidate source for the future development of possible oral phytoestrogenic nutraceutical. There are various factors to consider in the development of such a nutraceutical [29-32], however, two main preliminary concerns would be to firstly, identify the compound or compounds pertaining to the specific estrogenic activity of the extract and, secondly, to determine whether these compounds are absorbed intestinally, as it is required for a therapeutic effect [403-405]. In the next section the types of transport that occur during intestinal absorption

will be discussed. The focus will fall on the intestinal structure and function, phytoestrogen-related metabolism, and intestinal absorption of *Cyclopia* extract-related compounds. Furthermore, models for predicting and evaluating intestinal absorption will also be discussed.

2.6.1 Intestinal structure and function

The small intestine is known as the primary site of intestinal absorption of orally administered drugs [406] (Fig. 13a). The inner absorptive surface of the small intestinal wall is greatly increased by the presence of villi, whilst the colonic intestinal wall possesses invaginations, and as such contributes to increased absorption of drug molecules [407]. The intestinal epithelial cell membrane serves as the primary barrier in drug transport from the lumen to the blood (Fig. 13b). In the intestine, most lipophilic compounds permeate the intestine via a passive diffusion process. Diffusion across a mucosal layer can either take place via a paracellular (between cells) or transcellular (across cells) pathway [408]. Paracellular diffusion commonly occurs via tight junctions between two or three cells, whereas transcellular diffusion may occur either via receptor-mediated binding followed by vesicular transport (transcytosis), or via passive absorptive transcytosis (fluid phase) [409]. Compounds can also be absorbed by means of carrier-mediated transport, involving transporters such as H^+/peptide cotransporter, peptide transporter 1 (PEPT1), for movement into intestinal epithelial cells followed by active transport into the bloodstream by a transporter such as multidrug-resistance-associated protein 3 (MRP3). In contrast, some compounds make use of efflux transporters such as P-glycoprotein (P-gp) or breast cancer resistance protein (BCRP), resulting in a lowered bioavailability of these compounds as they are frequently pumped back into the lumen [410]. In addition, drug molecules may also be metabolised by intestinal enzymes such as cytochrome P450-dependent mixed-function oxidase 3A4 (CYP3A4), resulting in inactivation [411]. Another means of diffusion process classification also exists whereby permeation processes are divided into non-polar (partitioning into the lipid bilayer of the mucosa) and polar (aqueous channels in mucosa) pathways, however, literature suggests that the transepithelial permeation of most molecules occur as a simple first-order diffusion process [412,413].

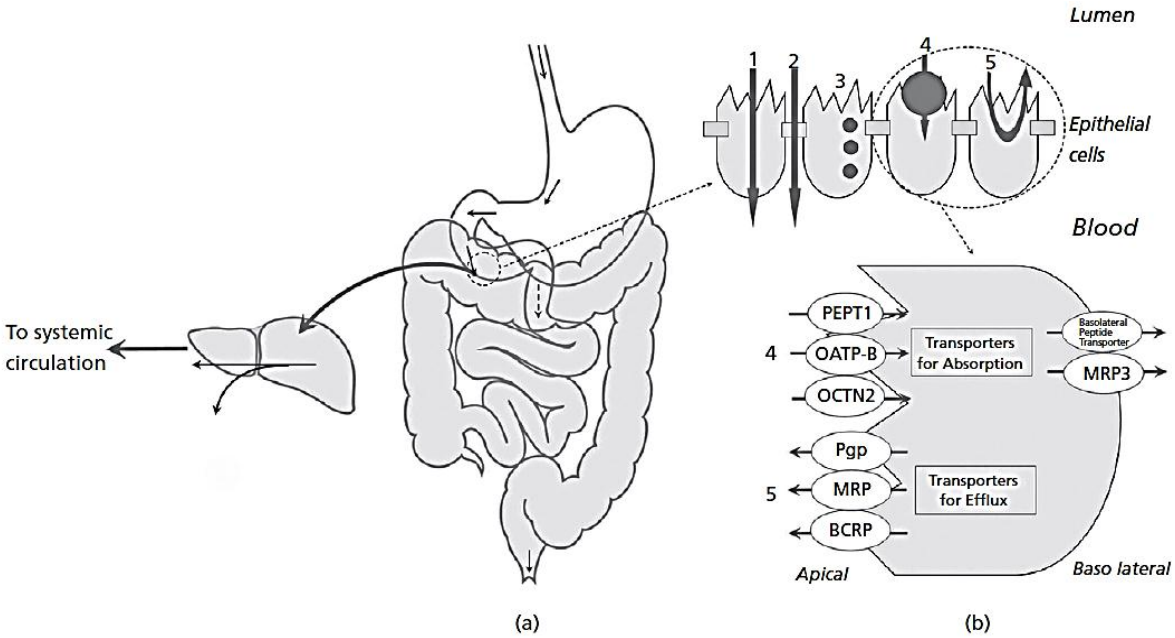

Figure 13 Small intestine physiological structure and function [403]**.** a) the oral drug absorption route, b) main pathways in the gastrointestinal tract absorption: 1) transcellular transport, 2) paracellular transport, 3) transcytosis (enzyme metabolism), 4) carrier-mediated transport and, 5) efflux transport. PEPT1 refers to H^+/peptide cotransporter 1, OATP-B refers to organic anion-transporting polypeptide-B, OCTN2 refers to novel organic cation transporter 2, Pgp refers to P-glycoprotein, MRP refers to multidrug resistance-associated protein, BCRP refers to breast cancer resistance protein and, MRP3 refers to multidrug resistance protein 3.

2.6.2 Intestinal metabolism and absorption of phytoestrogens

In the next section phytoestrogen metabolism and absorption will be discussed, and as lignans and isoflavones are considered to be the main phytoestrogens [414], and thus the most studied, the focus will mainly be on the metabolism and absorption of these plant constituents.

Isoflavones exist in three different forms in nature known as the acetylglycosides, aglycones (unconjugated) and malonylglycosides [415]. Daidzein and genistein are present in soybeans and soy products, mostly as their glycones, daidzin and genistin [416]. After ingestion, these glycones are hydrolyzed by intestinal β-glucosidases releasing the bioactive aglycones, genistein and daidzein, which enables intestinal absorption of these compounds [238,417]. Pharmacokinetic studies in humans have indicated that absorption of isoflavones occur along the entire length of the intestine [418]. The lignans, matairesinol and secoisolariciresinol, are converted into enterolactone and enterodiol, respectively, by bacterial metabolism, where after enterodiol may then be converted

into enterolactone in the gut [419]. Once absorption occurs, isoflavones and lignans undergo hepatic sulfation and glucuronidation, where after the conjugates either undergo enterohepatic circulation, or they are excreted in the bile and urine [417,420]. The formation of genistein and daidzein can also occur through bacterial demethylation of formononetin and biochanin A (found in red clover), respectively [421]. Thereafter, daidzein is metabolised to non-estrogenic *O*-desmethylangolensin (*O*-DMA) and estrogenic equol [422], whereas genistein is metabloised to dihydrogenistein and consequently 6´-hydroxy-*O*-DMA (6´-OH-*O*-DMA) [213,423]. There is, however, a considerable amount of interindividual variation in the metabolism of isoflavones [239]. For instance, 80-90 % of humans can convert daidzein to *O*-DMA, whereas only 30-50 % of humans can convert daidzein to equol [424,425]. The metabolites *O*-DMA and equol occur in an inverse relationship [426], and as equol is regarded as estrogenic, such variation may be of toxilogical importance [427]. The variation in phytoestrogen metabolism may be attributed to the presence or absence of bacteria adept at metabolising these compounds, the intestinal transition time, or the pH. These factors are influenced by the host diet, diseases, surgery, immunity and intake of drugs.

Excretion of isoflavones from the body occurs within 24 h after ingestion, predominantly in urine, and to a lesser extent, in feces [428]. It has been shown that genistein and daidzein urinary excretion content does not correlate with ingested doses, which might be attributed to the lessened absorption of isoflavones with high ingested doses [429]. Equol, genistein and daidzein have been detected in human urine [430], plasma [431], saliva, prostatic fluid and breast aspirate [432]. Contrastingly, lignan concentration in urine and plasma increases in a dose-dependent manner [433]. In addition, it has been shown that the lignans, enterodiol and enterolactone, are detected in human semen [434], bile [435], faeces and also serum [436]. It has been shown that various factors play a role in equol excretion, including individual genetic factors, diet, and type of intestinal bacteria present [124,437,438].

An important factor to keep in mind is the effect of the matrix (presence of other compounds) in which compounds are administered to the host, on the absorption of compounds of interest. For example, phloridzin, a constituent of apples, has been shown to amplify the absorption of the isoflavone genistin (genistein glucoside) in rat small intestine [439], furthermore, phytic acid has been shown to enhance the absorption of anthocyanins in both humans and rats [440]. In addition, milk has been shown to enhance absorption of green tea polyphenols in a Caco-2 cell model [441]. In contrast, the flavonoids, quercitin and rutin, attach to the small intestine of the rat in an *in vitro* assay, which greatly limits their absorption [442]. Furthermore, the flavonol quercetin-3-glucoside has been shown to inhibit cyanidin-3-glucoside absorption in a mouse *in vitro* assay [443]. The presence of other compounds do not always have an effect on the compound of interest's absorption, as demonstrated by Zuo *et al.* [444], where the Caco-2 cell model, rat *in situ* intestinal perfusion model, as well as the *in vivo* pharmacokinetics studies in rats demonstrated that the co-occurring components in a hawthorn phenolic extract might not have a significant effect on the intestinal absorption of the three major hawthorn flavonoids, hyperoside, isoquercitrin and epicatechin.

2.6.3 Intestinal absorption of *Cyclopia* extracts

No absorption studies have been performed on the *Cyclopia subternata* extract SM6Met, however, as the polyphenolic content of SM6Met has been investigated [26], available literature on the absorption of the individual compounds or extracts containing the same polyphenols known to be present in SM6Met can be investigated.

Previous qHPLC results have indicated that SM6Met contains numerous polyphenolic compounds [26,268]. These compounds include two xanthones, mangiferin and isomangiferin, two flavanones eriocitrin and hesperidin, two flavones, luteolin and apigenin-6,8-di-*C*-glucoside, and two unknown compounds, unknown 1 and unknown 2 [26,268]. Unknown 1 was later identified as the flavone, scolymoside, and unknown 2 was identified as the dihydrochalcone, phloretin-3',5'-di-*C*-glucoside

[268]. Furthermore, another dihydrochalcone, 3-hydroxyphloretin-3',5'-di-*C*-hexoside, referred to as aspalathin by Visser *et al*. [268], was also identified in SM6Met. In addition, the presence of two benzophenones, iriflophenone-3-*C*-glucoside and iriflophenone-di-*O*,*C*-hexoside, and also, the hydroxybenzoic acid, protocatechuic acid, was indicated in SM6Met [268].

In a study by Bock *et al*. [445] pigs were fed a mangiferin-enriched *C. genistoides* extract, whereby pigs were fed 1 mg hesperidin per kilogram bodyweight, and 74 mg mangiferin per kilogram bodyweight for 11 days, respectively, where after plasma, urine, and faeces samples were collected at various time intervals. The plasma samples revealed only the presence of the mangiferin aglycone, norathyriol, whereas the urine revealed six metabolites of mangiferin and hesperidin. Furthermore, 8.2 % of initial mangiferin intake was detected in the faeces, whereas no hesperidin or hesperidin metabolites were detected in the faeces.

In a study investigating absorption of the dihydrochalcone *C*-glucoside, aspalathin, an aspalathin-rich rooibos extract was orally administered to pigs at 157-167 mg aspalathin per kg body weight daily for 11 days, whereafter plasma and urine samples were collected at various time intervals [446]. Even though no traces of aspalathin was detected in pig plasma, five diydrochalcones were detected in the urine samples, indicating that absorption of aspalathin did take place, and that cleavage of the compound took place to form the aglycone and sugar moiety. This study also concluded that phase II enzyme metabolism was involved as the methylation and glucoronidation of metabolites occurred. In addition, a human study [447] also investigated bioavailability of aspalathin, nothofagin and also four eriodictyol-*C*-glycoside isomers. Upon administration of these compounds to five male and five female volunteers, where each had to consume 500 ml of fermented or unfermented tea, urine and plasma samples were collected at 0-24 h. The unfermented tea had 10 times higher aspalathin and nothofagin, and a four times lower eriodictyol-*C*-glycoside content, than fermented tea. Even though no metabolites were detected in the plasma, urine analysis revealed eight metabolites, of which the fermented drink yielded mainly eriodictyol-*O*-sulfate, and

the unfermented drink, *O*-methyl-aspalathin-*O*-glucuronide. Interestingly, this study concluded that all aspalathin metabolites were excreted within 5 h, indicating absorption in the small intestine, whereas the eriodictyol metabolites were excreted between 5-12 h, indicating absorption in the large intestine.

Furthermore, rat studies investigating the absorption of the dihydrochalcones, phloretin and phloridzin, was conducted by Crespy *et al*. [448]. Rats were fed a single meal containing 0.157 % of the dihydrochalcone aglycone, phloretin, and 0.22 % of the glucosidic form of phloretin, phloridzin, of which the phloretin and phloridzin were the equivalent of 22 mg phloretin. Thereafter, plasma samples were collected as various time intervals, and analysed. Conjugated phloretin was mostly detected in the plasma, however, some unconjugated phloretin was also detected, whereas no intact phloridzin was detected, suggesting that cleavage of the glucoside moiety from phloridzin occurs during absorption.

In an investigation of flavone absorption by rats, Shimoi *et al*. [449] administered 50 µmol/kg luteolin or luteolin 7-*O*-*β*-glucoside by gastric intubation to rats, with blood samples collected at various time intervals afterwards. Analysis of plasma revealed that free luteolin, luteolin conjugates and methylated luteolin conjugates were present, illustrating that some luteolin can escape the intestinal conjugation and hepatic sulfation/methylation. The same study also demonstrated that luteolin was transformed to it glucuronide forms during intestinal permeation.

In a study by Zhang *et al*. [450] the benzophenone iriflophenone-3-*C*-glucoside, together with other compounds present in a mango leave extract, was orally administered to KK-A^y mice for 8 weeks at two different concentrations. Iriflophenone-3-*C*-glucoside was found to be present in both the blood samples and the soleus muscle samples, which is indicative of the fact that absorption did occur.

In conclusion, as detection of ingested compound metabolites in blood or urine is indicative of systemic absorption, most of the above mentioned compounds were absorbed. It is, however,

important to remember that variability occurs in the absorption of orally administered compounds. A factor contributing to this variability is the site of absorption, i.e. the small or large intestine. Furthermore, deglycosylation of the ingested compounds vary and serves as a prerequisite for absorption in a compound-dependent manner. In the next section we will discuss the various types of models available for the purpose of predicting intestinal absorption.

2.6.4 Models for predicting intestinal absorption

Commonly used test systems for the investigation of intestinal absorption of compounds are listed in Table 3, where the type of system, the different models, the species of origin, and also the pros and cons of the models are presented. These commonly used test systems may be divided into five different system classifications namely, *in vitro*, *in vivo*, *in situ*, *ex vivo* and *in-silico* models.

2.6.4.1 *In silico* methods

In silico methodology refers to the use of computational or virtual screening to accurately predict intestinal permeation. These models make use of the molecular descriptors of compounds such as compound molecular size, lipophilicity, hydrogen bonding capacity, etc. in order to accurately predict absorption, and in doing so it serves as a high throughput (HT), and thus time-saving alternative to the conventional experimental studies.

A well-known *in silico* model for the quantitative prediction of intestinal absorption was proposed by Lipinski *et al*. [478] in 1997, known as Lipinski's "Rule of five". In this model poor intestinal permeation is predicted for compounds with a molecular weight more than 500, a cLogP (octanol-water partitioning coefficient) value greater than five, and for compounds with more than 10 hydrogen bond acceptors (HBA), and more than 5 hydrogen bond donors (HBD) [461]. In addition, quantitative structure and property relationship (QSPR) programs, such as the ADMET Predictor program, or GastroPlus™, which makes use of the physicochemical, spectroscopic, and chromatographic properties of compounds, have been used to predict human intestinal absorption [456,457,479,480].

Table 3 Summary of the pros and cons of commonly used assays for the prediction of intestinal absorption as adapted from Liu *et al*. [403].

System	Model	Species of origin	Pros	Cons
In silico	Computational approaches such as structure activity relationships	Computer databases	HT	1. Lack of active processes (at present) 2. Depends heavily on the quality and quantity of available data
In vitro	Artificial membrane systems (PAMPA, IAM)	—	1. HT 2. Easy to utilise and analyse	Dependent on lipid composition and pH
	Caco-2	Human colon adenocarcinoma	1. Most well established model 2. Differentiates and expresses relevant transporters 3. Moderate to HT 4. For active and passive transport	1. Expression of influx transporters is variable 2. Long culture period (21 days) 3. Can measure only transcellular permeation accurately
	MDCK	Dog kidney	Ideal for transfections	Cells express low levels of ABC transporters
	2/4/A1	Rat foetal intestinal epithelial cells	Ideal for paracellularly absorbed compounds	Temperature sensitive
Ex vivo	Rat everted gut sacs	Rats guts	1. Easy and inexpensive 2. Useful for mechanistic studies	1.Viability of tissue (<30 min)
	Flow-through diffusion	Porcine intestine	1. HT 2. Can be used to determine compound pharmacokinetic data	Produces a large amount of variation in results
In situ	Rat intestinal perfusion	Rat intestines	1.Transporters, enzymes 2. Relevant tight junctions	1. Difficult analysis
In vivo	Animal blood or urine	Rats, mice, rabbits, dogs, pigs	1. Transporters, enzymes present 2. Relevant tight junctions 3. Presystemic metabolism 4. Closer to human *in vivo* conditions	1. Ethical considerations 2. Expensive 3. Laborious and time-consuming

HT refers to high throughput, MDCK refers to Madin-Darby canine kidney, PAMPA refers to parallel artificial membrane permeability assay, IAM refers to immobilized artificial membrane

Most of the computational models currently in use only predict passive absorption as it does not account for influx or efflux transporters. As this technique can be used only for passively transported compounds, it may lead to inaccurate results regarding compounds that are also actively transported [481].

As computational modelling of intestinal absorption is still a relatively new field, researchers propose the combinatorial use of *in vivo* methods and *in silico* methods to accurately predict human intestinal absorption of a compound. This will also allow validation of the sole use of *in silico* methods for use in future experiments [481].

2.6.4.2 *In vitro* methods

The *in vitro* section will only focus on the most frequently used *in vitro* assay, the Caco-2 cell monolayer assay.

2.6.4.2.1 Caco-2 cell monolayer

The most commonly used *in vitro* approach for prediction of intestinal absorption is the use of the Caco-2 cell monolayer (human adenocarcinoma cells derived from the colon) [451-457] (Fig. 17).

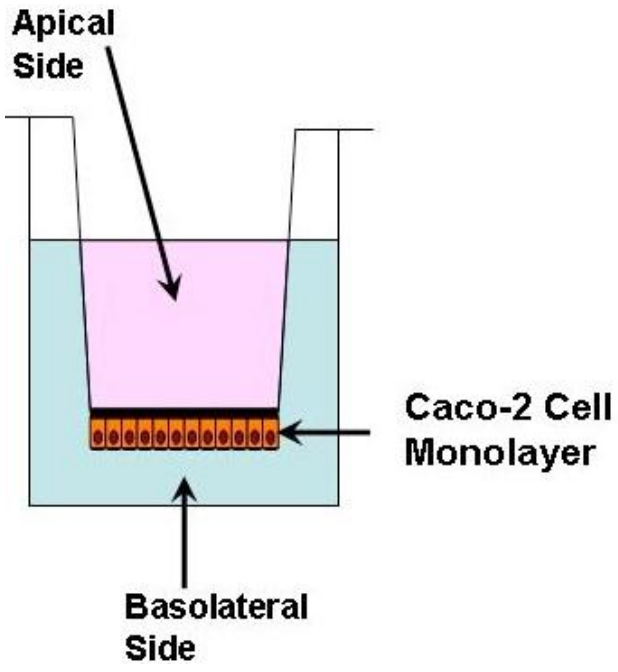

Figure 17 Graphical representation of the Caco-2 cell monolayer assay experimental setup.

As the colonic Caco-2 cells are morphologically and functionally similar to small intestinal cells, advantages of using these cells are that these cells differentiate and express relevant transporters, and as such, active and passive transport can occur. Also, the technique is known as a moderate to HT technique, as it yields a relatively large amount of data per experiment [403]. Factors that inhibit the performance of the cells include the lack of cell heterogeneity, and variable expression of only some intestinal enzymes and transporters [458,459]. Furthermore, the culture period of 21 days make the use of these cells a time-consuming pursuit. In a study by Walter *et al*. [460] it was shown that the permeability of the paracellular marker, mannitol, varied as much 100-fold depending on the source of the Caco-2 cells, however, regardless of its source these cells will always separate the completely absorbed compounds from the poorly absorbed compounds. Further limitations in the use of this cell line include poor predictive absorption of some compounds, for example, some low molecular weight compounds have been shown to be adequately absorbed in humans, whilst the Caco-2 model have predicted poor absorption for these compounds [461]. Furthermore, the use of an adequate amount of organic solvent in the cell model is restricted, as integrity of the cell junctions is easily disturbed, even at a low percentage concentration of these solvents [461].

2.6.4.3 *Ex vivo* methods

Ex vivo methods includes the excision of the intestinal area of interest e.g. the jejunum, and investigation of the tissue specimen outside the body of the animal of origin. Examples of two well-known *ex vivo* absorption methods are the everted intestinal sac assay and the flow-through diffusion assay.

2.6.4.3.1 The rat everted intestinal sac assay

The rat everted (definition: the position of being turned outward) intestinal sac assay developed by Wilson *et al.* [476], whereby the everted sacs are filled with incubation solution, sealed at both ends, and put into a solution containing the investigated drug, in order to determine the presence and extent of the intestinal permeation for the drug. Kelber *et al.* [477] showed that this technique

can yield results that correlate accurately with the Caco-2 cell monolayer assay and *in vivo* assays. Limitations of this technique include the absence of *in vivo* conditions such as blood and nerve supply, and the absence of intestinal microflora that can play a part in the intestinal absorption of some compounds.

2.6.4.3.2 Flow-through diffusion system

The flow-through diffusion system (Fig. 18) is useful for predicting permeation of a compound, or comparing the permeation of compounds through a mucosal sample, since the structural integrity of the tissue specimen is close to that of the *in vivo* conditions. However, even though the technique yields seven replicates per experiment, and the experiment only takes 24 h, a large amount of variation exists between replicates, and as such the researcher needs to perform the experiment multiple times, resulting in the analysis of many collected fractions, in order to get a viable or significant result. The flow-through diffusion assay, however, when used for purposes of predicting human intestinal absorption, incorporates the use of either porcine small or large intestine, which is useful as porcine intestine resembles the human intestine both functionally and morphologically [462]. In addition, the flow-through diffusion assay allows pharmacokinetic evaluation of the absorption of compounds.

The method has been used to investigate various mucosal membranes for the permeability of drugs. In a study by van der Bijl *et al*. [463], the flow-through diffusion system was used to compare human buccal and human vaginal mucosa permeability for sumatriptan, a synthetic drug used in the treatment of migraine headaches. This study was later extended to compare the permeability of various chemical markers through human buccal and vaginal mucosa, as well as porcine mouth floor and buccal mucosa [464].

Figure 18 The flow-through diffusion assay system set-up. On the bottom left of the figure is the fraction collector. Above the fraction collector are seven flow-through chambers, each containing a porcine tissue sample with the apical-side facing upwards. The sample is placed on the porcine intestine, and phosphate buffered saline (PBS) is pumped through the flow-through chamber containing the intestinal sample by means of a pump present on the bottom right of the figure.

In addition, the flow-through diffusion technique was used to investigate the effects of penetration enhancers on the permeation of human corneal permeability for the immunosuppressive agent, Cyclopsporin A [465]. It was also used to compare the permeability of human and rabbit cornea for Cyclosporin A and tritiated water [466]. More recently, a comparative diffusion study of drugs through porcine bronchial tissue was performed by Van Zyl *et al*. [467].

Permeability of human intestinal mucosa has also been evaluated using this technique [468], where it was concluded that the continuous flow-through perfusion system was sufficient for testing intestinal permeation of compounds with a molecular weight smaller than 500 Da, however other human mucosa, such as vaginal mucosa, have to be considered as an alternative to intestinal mucosa for intestinal permeation evaluation of compounds larger than 500 Da. Furthermore, in 2003 a comparative permeation study was performed to investigate permeation of a range of compounds through human vaginal, colonic and small intestinal tissue [469]. It was concluded that colonic

mucosa is more permeable to E_2, water, arecoline and arecaidine, however, it was found that drugs with a molecular weight of more than 300 Da may necessitate the use of other mucosal membranes, such as vaginal mucosa, as an alternative.

2.6.4.4 *In situ* methods

The *in situ* rat single pass intestinal perfusion model (Fig. 19) includes anesthetising the rat of interest, followed by creating two incisions, one at the top and one at the bottom of the still intact intestinal area of interest (the jejunum as in the case of Fig. 19). The inlet of the tube is inserted at the proximal incision, whereas the outlet of the tube exits by the distal incision. Perfusion solution containing the investigated drug is then pumped through the tubing with samples collected at the outlet (Fig. 19), where after the perfusate is analysed in order to determine the absorption of compounds. The *in situ* perfusion model is a dependable assay as it devises biliary secretion profiles that resemble *in vivo* conditions, and it incorporates transporters and enzymes present in *in vivo* conditions [403].

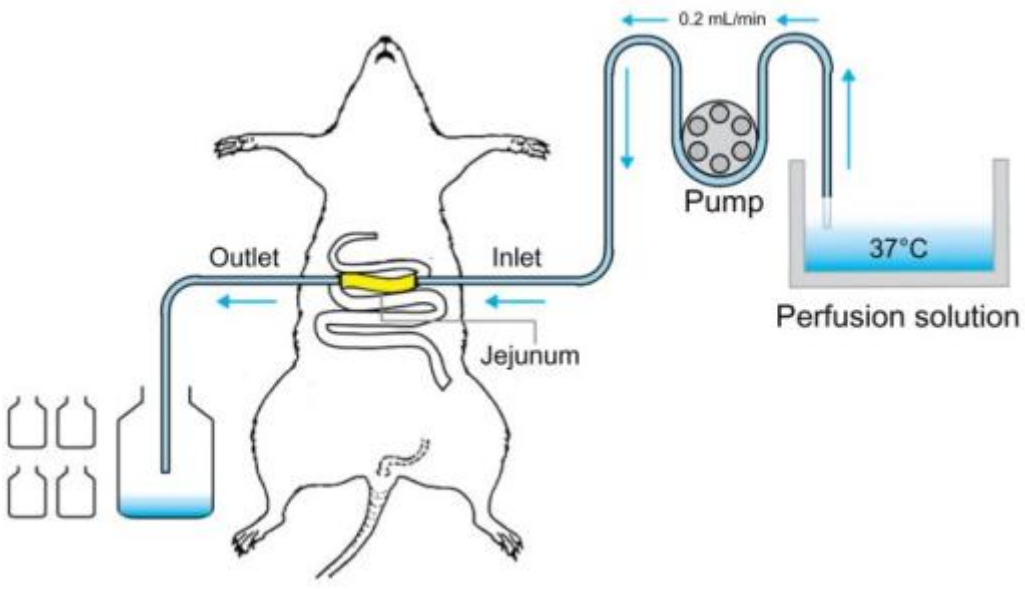

Figure 19 Graphical representation of the rat single pass intestinal perfusion model [473]**.**

In order to elucidate the absorption mechanism, absorption site and transport route, the *in situ* method can be combined with the Caco-2 method as was done in Zhou *et al.* [474]. An advantage

that the *in situ* technique holds over an *in vitro* technique is the incorporation of the intact nerve and blood supply. Various different types of *in situ* perfusion has been investigated such as oscillating perfusion, the common single pass perfusion, closed loop perfusion, and studies have even been extended to humans as in Lennernas *et al*. [475], where this technique has been dubbed accurate in the prediction of passively transported compounds. In contrast, the single pass perfusion method has some limitations, namely it is not cost-effective, and the rate of diffusion from the luminal side does not always accurately correlate with the rate of actual absorption [461].

2.6.4.5 *In vivo* methods

In vivo models are frequently used in the prediction of human intestinal absorption of herbal medicines, individual compounds, or extracts as mentioned in the section on absorption of polyphenols also present in SM6Met. Advantages of the use of an *in vivo* model to predict intestinal absorption includes the incorporation of enzymes, transporters, and relevant tight junctions. Furthermore, *in vivo* test systems also incorporate the use of a presystemic metabolism, and as such, *in vivo* test system conditions are the closest to human *in vivo* conditions (Table 3). The animal model regarded as the most accurate in predicting human intestinal absorption is the pig. However, a few disadvantages exist, namely the requirement of a large number of animals, inter-animal variability, and also the long period usually needed for a study. Drug absorption has been shown to be influenced by the age, sex, chronic therapy, and disease state [470,471] of the animal. In addition, the absorption results can be species-dependent [472], and as such caution should be taken in explaining and extrapolating results. Species-dependent absorption can be ascribed to fluctuations in physiological factors within species, such as differential distribution of enzymes, gastrointestinal tract motility, pH, and transporters [426]. Even though *in vivo* methods are resource-intensive, it is commonly used to predict the absolute extent of human intestinal drug absorption [426].

In summary, numerous test system types and also test system type models are available to predict human intestinal absorption. Some models are more useful than others, however, most experimenters will use the model that presents the highest throughput, and yields the most reliable data. For the purpose of a preliminary investigation of the absorption of compounds present in an extract such as SM6Met, *ex vivo* and *in vitro* assays, such as the flow-through diffusion assay and the Caco-2 assay, would most frequently be used. However, in an attempt to more closely predict human intestinal absorption one should incorporate the use of as many as possible other test systems as it entails a more holistic approach.

2.7 Conclusion and aim of thesis

Conventional HRT used in the treatment of menopausal symptoms and menopause related diseases has been shown to be effective in most instances; however, its usage is accompanied by a range of side effects [6-18], of which invasive breast cancer is considered the primary adverse outcome [9,10]. In the search for a safer, more natural alternative to conventional HRT women have shown an interest in phytoestrogens [191,192], perceived to have a better side effects profile [153,156].

In 2007 Verhoog *et al.* [24] identified two *Cyclopia* species displaying phytoestrogenic properties using ER binding assays. It was also concluded that unfermented rather than fermented plant material should be used for extracts to display optimal estrogenic activity, and that methanol rather than water should be used as extraction solvent to obtain an extract that displays optimal estrogenic activity. Verhoog *et al*. [25] did a follow-up study in 2007 focussing on *C. genistoides* and showing that its extracts displayed phytoestrogenic properties and acted predominantly via ERβ, however, none of the investigated polyphenols could explain this activity. In 2008 Mfenyana *et al*. [26] screened various *C. genistoides* and *C. subternata* extracts for estrogenic activity in order to determine the most potent estrogenic extract, which was identified as the *C. subternata* extract, SM6Met. In 2013 Visser *et al*. [268] showed that SM6Met has three desirable estrogenic traits, namely ERα antagonism, ERβ agonism, and the ability to antagonise E_2-induced breast cancer cell

proliferation. In addition, it was shown that P104, a *C. genistoides* DME, binds to both ERα and ERβ, but transactivates only via ERβ in an ERE-containing promoter reporter assay, and that even though it induces breast cancer cell-proliferation, it does so at significantly lower levels than E_2. These attributes displayed by P104 and SM6Met are desirable as, unlike conventional HRT, they should not increase the risk of breast cancer, and as such, should be retained in a future oral phytoestrogenic nutraceutical, however, as no more P104 plant material is available for further investigation, SM6Met serves as the candidate for the current study.

The current study, part of a comprehensive study on the phytoestrogenic activity of *Cyclopia*, investigates a specific *Cyclopia* extract as a candidate source for the possible future development of an oral nutraceutical for the treatment of menopausal symptoms. The study will focus on two aspects that are important within this context, namely the identification of specific compounds conferring the desired estrogenic traits, and also whether these compounds will be absorbed intestinally. Thus the aims of this study are firstly, activity-guided fractionation of SM6Met in order to identify compounds conferring specific estrogenic traits to SM6Met, and secondly to evaluate intestinal absorption of SM6Met compounds. For the first aim SM6Met will be separated and fractionated into less complex extracts and fractions. After every fractionation step *in vitro* promoter reporter and breast cancer cell proliferation assays will be employed to monitor the estrogenic activity. Also, qHPLC will allow quantitative identification of compounds in extracts or fractions to link the presence of compounds with a specific estrogenic activity. For the second aim, the permeability of porcine small and large intestinal tissue for SM6Met compounds will be investigated using the *ex vivo* flow-through diffusion assay. Analysis of collected fractions will be performed using liquid chromatography–tandem mass spectrometry (LC-MS/MS) in an attempt to predict absorption of SM6Met compounds in the human body. Results obtained from the flow-through diffusion assay will also be accompanied by results obtained from various *in silico* models in an attempt to more accurately predict human intestinal absorption.

2.8 Bibliography

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Chapter 3
Activity-guided fractionation of *Cyclopia subternata* extract, SM6Met

3.1 Introduction

Menopause is characterised by a decrease in ovarian function and associated estrogen production [1], which in some women leads to adverse side effects [2]. Symptoms are known to be more pronounced after ablation of the ovaries as per oophorectomy [3,4]. Menopausal symptoms may be divided into climacteric symptoms, such as hot flushes and night sweats, and psychosomatic symptoms, such as poor concentration, memory loss and loss of confidence [5]. Menopause may also cause long-term problems such as osteoporosis, CHD and Alzheimer's disease [6-8].

Menopausal symptoms are traditionally treated with HRT, which supplements the deficit of estrogen [6-9]. Specifically, HRT consist of estrogen, for women without an intact uterus, or estrogen and progestin, for women with an intact uterus [10]. HRT may reduce the risk of CHD, osteoporosis, fatal colon cancer [11] and possibly Alzheimer's disease [8,12-15], while preventing a decline in cognitive function [16], and improving quality of life [17]. However, HRT usage results in several adverse effects such as an increased risk of breast, ovarian and endometrial cancer [18-24], vaginal bleeding [25], strokes, venous thromboembolism [26], cardiovascular disease [3,27,28], and gall bladder disease [29], of which invasive breast cancer is considered the primary adverse outcome [27,28].

The adverse side effects profile of conventional HRT has prompted the search for alternatives with a safer side effect profile, specifically, a treatment that would effectively address menopausal symptoms, while as a side effect preventing breast cancer [30,31]. Understanding the molecular mechanism of action of estrogen could aid in this search. At the molecular level, in most tissue types the action of estrogen is mediated via the ER, which forms part of a large superfamily of nuclear receptors, and functions as a ligand activated transcription factor [32]. The two mains subypes of the ER are ERα and ERβ [33-35]. Estrogen activates both ER-subtypes [36,37], however, ER-subtype specific agonist and antagonist ligands have been identified [38-43]. Physiologically, ERα is associated with an increase in cellular proliferation, whereas ERβ inhibits

ERα-mediated cell proliferation [44], thus it has been suggested that compounds, which antagonise ERα and/or activate ERβ may be useful in the prevention and treatment of breast cancer [45-47].

Phytoestrogens are plant compounds that mimic the action of estrogen as they are structurally and/ or functionally similar to mammalian estrogen and its active metabolites [48] and thus may serve as a possible alternative to conventional HRT [49,50]. Furthermore, studies have shown that although most phytoestrogens bind both ERα and ERβ, they generally show a higher binding affinity for ERβ than ERα [35,51-53]. In addition, epidemiological studies have shown that phytoestrogen consumption, in Asian populations as part of a soy-based diet, correlates with a low incidence of prostate, colon and breast cancers [54,55]. Phytoestrogens are thus seen as a safer alternative to conventional HRT [56] and may serve as a source of ER subtype specific ligands [57].

The genus *Cyclopia* (family: Fabaceae), which is endemic to the Western and Eastern Cape provinces of South Africa [58], contains phytoestrogenic compounds [59]. The aerial parts of the bush have been and are still used for the production of honeybush tea, which has a sweetish flavour and dark-brown colour [60]. DMEs from *C. genistoides* and *C. subternata* have been shown to bind the ER and transactivate an ERE-containing promoter reporter construct [61-63]. Whereas initially only the DME from *C. genistoides* (P104) was investigated for ER subtype specific estrogenicity and found to activate only via ERβ [61,62], a recent study has established that DMEs from both *C. genistoides* (P104) and *C. subternata* (SM6Met) are ERβ agonists and ERα antagonists [64]. In addition, studies have shown that even though *Cyclopia* extracts, including SM6Met and P104, induce weak proliferation of a human breast cancer cell line (MCF-7BUS), they antagonise estrogen-induced proliferation [62-64]. These results suggests that DMEs of *Cyclopia* may serve as a possible source of ER subtype specific ligands for the development of a phytoestrogenic nutraceutical that may be suitable for the treatment of menopausal symptoms, while preventing the development of breast cancer [64].

The current study, part of a larger study investigating the development of a phytoestrogenic nutraceutical from *Cyclopia*, focusses on activity-guided fractionation of the DME from *Cyclopia subternata*, SM6Met. SM6Met was chosen for this study as it is the only extract investigated for which the specific source material used in the previous studies was available in bulk. Our activity-guided fractionation will concentrate on the positive estrogenic attributes previously identified for SM6Met, namely ERβ agonism, ERα antagonism and antagonism of estrogen-induced breast cancer cell proliferation [62-64]. Activity-guided fractionation is being pursued in an attempt to identify the compound or compounds responsible for the above mentioned estrogenic activity. Such a marker compound(s) would be useful not only for quality control of a future nutraceutical, but also to screen prospective *Cyclopia* harvestings destined for nutraceutical production. In addition, such a marker compound(s) could in future be used to direct plant breeding programs. Thus, qHPLC and LC-MS/MS were used to track polyphenols during fractionation for correspondence with above-mentioned positive estrogenic attributes.

3.2 Materials and Methods

3.2.1 Fractionation of SM6Met

3.2.1.1 Large scale SM6Met production

The method (Fig. S1), executed at room temperature and adapted from Mfenyana *et al.* [63], entailed defatting of 500 g finely milled, dried plant material, originating from a M6 *Cyclopia subternata* harvesting (harvested 30 March 2004 near Barrydale, South Africa) [63], by stirred extraction over a period of 24 hours, using 2 L of dichloromethane (Merck®, 99.0% pure). Following extraction the plant material was filtered, the filtrate discarded, and the dichloromethane-extracted M6 air-dried overnight at room temperature. This defatting process was repeated four times. Next the air-dried, defatted M6 plant material (400-500 g) was subjected to sequential extraction using three solvents (2 L each) in order of increasing polarity (ethyl acetate (99.5% pure), ethanol (99.5% pure) and methanol (99.0% pure), supplied by Merck®, South Africa). Each

extraction step in the sequence was performed three times for 3 hours per extraction. The filtrates of the methanol extraction step was retained and pooled. Before a solvent change was made, the plant material was air-dried overnight in a fume cabinet at room temperature. The methanol was evaporated under vacuum (Büchi Rotavap®, Switzerland) at 40°C from the pooled extract, whereafter the resultant extract was freeze-dried (Virtis Advantage Plus®, USA). The resultant DME was ground with a pestle and mortar until a fine powder was obtained, which was then stored under vacuum in a desiccator in the dark at room temperature. Four batches of SM6Met (B1-B4) were prepared using the protocol above with B1-4Mix being a 1:1:1:1 homogenised mixture of the batches B1-B4.

3.2.1.2 Liquid-liquid fractionation

SM6Met B1-4Mix (4.3 g), suspended in 300 mL deionised water and decanted into a separating funnel, was liquid-liquid extracted using 150 mL of *n*-butanol (BuOH) (Sigma Aldrich®, South Africa). The mixture was then shaken lightly and inverting 5 times, and releasing the pressure in the separating funnel after every invert. After extraction the mixture was allowed to form two layers, and the lower, polar, and the upper, less polar, layers collected separately. For the purpose of this study the less polar fraction will be referred to as non-polar. The lower layer was then decanted back into the separating funnel, 150 mL of fresh n-butanol added, and the liquid-liquid extraction procedure repeated until four n-butanol partitionings were done in total and the respective fractions pooled. The resultant fractions, polar fraction (PF) and non-polar fraction (NPF), were rotary evaporated under vacuum at 40°C, where after the extracts were frozen at -20°C overnight. Following freeze-drying, the dried extracts were stored at room temperature in the dark under vacuum in a desiccator.

3.2.1.3 High performance counter-current chromatography (HPCCC) fractionation

The instrument used was a multilayer coil planet J-type centrifuge Spectrum model (Dynamic Extraction, United Kingdom). The machine is equipped with two preparative (1.6 mm i.d.) and two

analytical (0.8 mm i.d.) coils of polytetrafluoroethylene (PTFE) tubing, connected in series for a total volume of 172 mL. The inner β_r-value for the preparative coil was measured as 0.52 at the internal end of the coil and the outer β_r-value was 0.86 (equation $\beta_r = r/R$, in this case r is defined as the distance from the coil (planetary) axis to the nearest and farthest layer of the PTFE tubes wound around the coil system), while the inner and outer β_r-values for the analytical coil was 0.64 and 0.81, respectively. Fractionation was performed at 30°C, a rotation speed of 1600 revolutions per min (RPM) and a flow rate of 3 mL/min.

The two-phase solvent system used consisted of *tert*-butyl methyl ether (Sigma Aldrich®) – butanol (Sigma Aldrich®) – water (2:1:5, v/v). The solvents were mixed in a separating funnel, allowed to equilibrate at room temperature and separated into the organic and aqueous phases shortly before use. The separated phases were degassed using sonication. The upper organic phase served as the stationary phase, whereas the lower aqueous phase served as the mobile phase.

The coiled column was filled with the stationary (organic) phase using a Gilson® 305 HPLC pump (Gilson®, USA), equipped with a Gilson® 806 manometric module. The sample (30 mg NPF) was dissolved in 2.5 mL organic phase and 2.5 mL aqueous phase and then injected into the coil using a manual sample injection valve and a 10 mL loop. The mobile (aqueous) phase was pumped in the head-to-tail direction at a flow rate of 3 mL/min. The effluent of the column, monitored between 210 nm and 400 nm, using a Waters® 2996 diode-array detector (Waters®, USA) equipped with a semi-preparative flow cell (3 mm path length), was collected into test tubes at 1 min intervals, using a Gilson® FC203B fraction collector. The mobile phase flow was stopped at 52 min and the stationary phase pumped at 10 ml/min until 75 min. The procedure was repeated 15 times processing 450 mg NPF in total. The fractions collected were pooled into 3 main fractions, F1-F3, based on clustering of maximum absorbance peaks seen at 210 nm to 400 nm in separation of the NPF (Fig. S2). F1-F3 were rotary evaporated under vacuum and freeze-dried. The fractions were then stored at room temperature in the dark under vacuum in a desiccator.

3.2.2 Evaluation of estrogenic activity

3.2.2.1 Cell culture

HEK293 human embryonic kidney cells [65] (a kind gift from R. Louw, Stellenbosch University) and MCF-7BUS cells [66] (a kind gift from A. Soto, Tufts University, USA) were maintained in Dulbecco's Modified Eagles's Medium (DMEM) [Sigma Aldrich®] supplemented with 5% fetal calf serum (FCS) [Highveld Biologicals, South Africa], 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich®, South Africa). All cells were maintained at 37°C, 95% CO_2 and 5% relative humidity.

3.2.2.2 Compounds

The following compounds were used in cell culture for estrogenic assays: E_2 (17β-estradiol) (Sigma Aldrich®), luteolin (Extrasynthese®, France), mangiferin (Sigma-Aldrich®), isomangiferin (Chemos GmbH®, Germany), protocatechuic acid and *p*-coumaric acid (Fluka™ Analytical, Sigma-Aldrich®). Pure scolymoside and iriflophenone-3-*C*-glucoside could not be obtained at the time and thus enriched HPCCC fractions were used. The scolymoside (Fig. S3, Table S1) and iriflophenone-3-*C*-glucoside (Fig. S4, Table S2) enriched fractions were kind gifts from Dr. E. Willenburg, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa. As the concentration of the compounds present in these enriched fractions were not known at the time of experimental procedures, we presumed that the fractions represented a high percentage of scolymoside and iriflophenone-3-*C*-glucoside, respectively, thus, we treated the fractions as pure compounds. However, HPLC results later revealed that, even though the extracts displayed relatively high purity, scolymoside and iriflophenone-3-*C*-glucoside only represented ca. 21% and 31%, respectively, of the enriched fractions (Figs. S3&4, Tables S1&2).

3.2.2.3 Characterisation of cell lines used to evaluate estrogenic activity

3.2.2.3.1 Western blots

To investigate the presence of GR (glucocorticoid receptor), ERα, ERβ, PRA (progesterone receptor isoform A), PRB (progesterone receptor isoform B), MR (mineralocorticoid receptor) and AR (androgen receptor), HEK293 cells were seeded into 10 cm cell-binding sterile tissue culture plates (Corning®, USA) at a density of 4 x 10^6 cells per plate using 1:1 DMEM:HamF12 medium (Gibco® by Life Technologies, USA) containing 5% FCS and 100 IU/ml penicillin and 100 µg/ml streptomycin, where after the cells were incubated (95% relative humidity and 5% CO_2 at 37°C) for 24 h. The next day the medium was changed to DMEM containing 10% FCS,100 IU/ml penicillin and 100 µg/ml streptomycin and the cells washed once using phosphate buffered saline (PBS) preheated to 37°C. Twenty-four hours later the plates were placed on ice and washed once using 10 mL ice cold PBS. Cells were then lysed using 1225 µL lysis buffer A [10 mM KCl (Saarchem®, South Africa) 0.1% NP-40 (Roche®, South Africa), 10 mM Hepes (Sigma Aldrich®) pH 7.5, 1.5 mM $MgCl_2$ (Merck®) and Complete Mini protease inhibitor cocktail (Roche®)], kept on ice while placed on an orbital shaker for 10-20 mins and then frozen overnight at -20°C. The next day the lysate was thawed on ice, where after it was centrifuged at 12000 x g for 10 min at 4°C. The cleared lysate (supernatant) was then transferred to a 2.0 mL microfuge tube and stored at -20°C.

Lysates were electrophoresed at 200 V for 50 min using a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and proteins were transferred to a Hybond-ECL (AEC Amersham Biosciences®, South Africa) nitrocellulose membrane at 0.18 A for 90 min using a Bio-Rad® (USA) cell in transfer buffer (0.37 % SDS, 200 mM glycine, 240 mM Tris-HCL, 20 % (v/v) methanol). The membranes were then blocked for 120 min in either 5% (w/v) casein or 10% (w/v) milk powder dissolved into Tris-buffered saline (TBS) containing 0.1 % (v/v) Tween (TBST) at 25°C. Membranes were then washed once using TBST, where after the primary antibody was added to the membrane and incubated overnight at 4°C. Mouse anti-AR (sc-7305), mouse anti-

GAPDH (sc-47724) and rabbit anti-MR (H-300), anti-GR (H-300), anti-ERα (MC-20) and anti-ERβ (H-150) were all obtained from Santa Cruz Biotechnology®, USA. Mouse anti-PR (ab2764) was obtained from Abcam®, USA.

The next day the membranes were washed for 20 min, and then twice for 5 min on an orbital shaker at 25°C using TBST. Incubation of the secondary antibody (Horse Radish Peroxidase (HRP) – conjugated) followed for 2 h, whilst shaking at 25°C. Thereafter washing was performed as above, with an additional TBS rinse step, followed by a TBS wash for 5 min at 25°C. Secondary antibodies (HRP-conjugated goat anti-mouse (sc-2005) and HRP-conjugated goat anti-rabbit (sc-2030)) were obtained from Santa Cruz Biotechnology®. Visualisation was performed using above mentioned secondary antibodies, ECL Western blotting detection reagents (Pierce®, Thermo Scientific®, South Africa) and medical x-ray film (Axim) in conjunction with a molecular weight marker (PageRuler Prestained Protein Ladder, Thermo Scientific®) to determine if the bands present on the x-ray film correlate with the expected molecular weight of a specific nuclear receptor. For all experiments, except for probing of MR, untransfected COS-1 cell lysates were used as a negative control. For investigation in the presence of MR, untransfected Ect1/E6E7 cell lysates were used as a negative control. COS-1 cells transfected with a plasmid expressing the nuclear receptor of interest served as a positive control throughout.

To investigate the presence of ERα and ERβ in MCF-7BUS cells, the cells were seeded at a density of 2 x 10^6 cells per 10 cm tissue culture plate in the same temperature, humidity and CO_2 concentration as above mentioned for HEK293 cells. In the determination of the presence or the abscence of ERα and ERβ in MCF-7BUS cells the concurrent steps were then followed as for HEK293 cells using the same ERα and ERβ antibodies, and, untransfected COS-1 cell lysate served as a negative control.

3.2.2.3.2 Whole cell binding

HEK293 cells were seeded into 24-well cell-binding tissue culture plates (Corning®) at a density of 5 x 10^4 cells per well in 1:1 DMEM:HamF12 medium containing 5% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin where after the cells were incubated for 24 h. The following day the medium was aspirated and changed to DMEM with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. The next day the cells were washed 3 times using pre-heated PBS and then whole cell binding of the ER was performed using 20 nM radio-labelled estrogen (2,4,6,7-[^{3}H]17-β-oestradiol, specific activity 100 Ci/mmol, obtained from Amersham Biosciences®). The total binding mixture contained ^{3}H-E_2 and dimethylsulfoxide (DMSO), while the non-specific binding mixture contained ^{3}H-E_2 and 10^{-5} M unlabelled estrogen. Cells were then incubated for 4 h, where after the cells were washed 3 times (15 min per wash) using ice cold 0.2% PBS-BSA (bovine serum albumin obtained from Sigma Aldrich®). After the washing steps were completed, the cells were washed 3 times with pre-heated PBS in order to remove albumin and 100 µL passive lysis buffer (10% (v/v) glycerol (BDH Chemicals Ltd®, England), 0.2% (v/v) Triton (BDH Chemicals Ltd®), 2.8% (v/v) Tris-phosphate EDTA and 1.44 mM EDTA (Saarchem®, South Africa)) was added per well, the plates shaken at room temperature for 10-20 min and frozen overnight at -20°C. The next day, samples were thawed and 5 µL of lysate was used for protein determination according to the Bradford method [67]. The lysate that remained was transferred to scintillation vials containing 1 mL of scintillation fluid (Quickszint FLOW 2, Zinsser Analytic®, South Africa). Counting efficiency was 43% and all binding experiments showed a ligand depletion index of less than 10%. Radioactivity was measured using the Beckman® LS 3801 Beta-scintillation counter (Beckman®, South Africa) and results were normalised to protein content. Specific binding values were obtained by subtracting the non-specific binding values from the total binding values.

3.2.2.4 Promoter reporter studies

HEK293 cells were seeded at 4 x 10^6 cells per plate in sterile cell-binding 10 cm plates with 1:1 DMEM:HamF12 containing 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin, where

after the cells were allowed to settle for 24 h. The cells were then rinsed with PBS, pre-heated to 37°C, to remove the phenol red. The medium was then changed to phenol red-free DMEM supplemented with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. After the medium was changed the HEK293 cells were transfected with a total amount of 6150 ng of plasmid DNA. Transfections of cells were performed using FugeneXtreme transfection reagent (Roche®) according to manufacturer instructions. DMEM without phenol red (pre-heated to 37°C) was used in the initial DNA-FugeneXtreme-mixture. The complex was then added to the 10 cm plates containing the HEK293 cells (post–initial medium change), where after the cells were incubated for 24 h. Transfection optimisation was performed (Fig. S5) where after 150 ng pSG5-hERα [68] (a gift from F. Gannon, European Molecular Biology Laboratory, Heidelberg, Germany), 3750 ng ERE.vit2.luc [69] (a gift from K. Korach, National Institute of Environmental Health Science, USA) and 2250 ng empty vector (pGL2-Basic, Promega®, USA) were used for cells transfected with hERα. For the cells transfected with hERβ, 150 ng pSG5-hERβ [69] (a gift from F. Gannon), 3000 ng ERE.vit2.luc [69] and 3000 ng empty vector (pGL2-Basic) were used.

The next day the cells were replated into sterile 24-well cell-binding tissue culture plates at 5 x 10^4 cells per well using phenol red-free DMEM supplemented with 10% charcoal stripped FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. The following day the cells were induced with the respective test compounds or test samples, all dissolved in DMSO to yield a final DMSO concentration of 0.1% (v/v) in the medium. All extracts and fractions were tested at a concentration of 9.8 µg/mL as a previous experiment performed in the laboratory (data not shown) showed that this is the highest concentration of SM6Met readily soluble in DMSO. It would have been helpful to include dose response curves for all of the obtained extracts and fractions in order to obtain efficacy and potency values, which would have been useful in selecting specific treatment concentrations, however, we did not have sufficient amounts of sample available to perform these experiments. In addition, sufficient time to make more of the samples was not available as it is a time-consuming task. Efficacy and potency data regarding previously produced SM6Met, however, is available [63].

For investigation in agonist mode the extracts or test compounds were administered alone, whereas for investigation in antagonist mode, extracts or test compounds were administered in the presence of E_2 (10^{-11}M). DMSO served as the negative control as it was used to dissolve the compounds, whereas E_2, the endogenous ligand of the ER, served as the positive control. After 24 h the cells were rinsed using 500 µL ice cold PBS and lysed overnight at -20°C using 50 µL passive lysis buffer (as used for whole cell binding). Luciferase assays were performed using the Luciferase assay reagent from Promega®, USA according to manufacturer's instruction, where 50 µL of luciferase reagent was added to 10 µL of cell lysate. The luciferase activity, given in relative light units (RLUs), was obtained using a Veritas® luminometer (Turner Biosystems®, USA). Protein determination was done using the Bradford method [67], where 250 µL of the Bradford reagent was added to 5 µL of the cell lysate in a 96-well plate. Luciferase RLUs were normalised to protein concentration and the results were expressed as fold-induction compared to DMSO set as 1.

3.2.2.5 Proliferation studies

MCF-7 BUS cells, which had been withdrawn from the penicillin-streptomycin mixture for at least 7 days, were plated at a density of 10000 cells per well in a 96-well plate (Greiner Bio-One®, Germany) in DMEM containing 5% FCS. The next day the cells were washed with PBS (200 µL/well), pre-heated to 37°C, and the medium was changed to unsupplemented phenol red-free DMEM (200 µL/well) for 24 h (steroid and growth factor starvation) in the absence of the penicillin-streptomycin mixture. The treatment time schedule was optimised (Fig. S6), and thus, on day three the medium was aspirated and cells incubated for 24 h with test compounds or extracts (in DMSO) in phenol red-free DMEM containing 10% stripped FCS, in the absence of the penicillin-streptomycin mixture. The medium was then aspirated and cells re-induced at 24 and 48 h after initial treatment. All assays included a negative control consisting of 0.1% (v/v) DMSO and a positive control (10^{-9}M E_2). On day seven (24 h after the last treatment) the MTT (Sigma Aldrich®) assay was performed, where medium was aspirated and cells incubated with phenol red-free medium (200µL/well) in the absence of the penicillin-streptomycin mixture and stripped FCS, plus

MTT solution (5 mg/mL; 50 µL/well) for 4 h at 37°C. After incubation, the wells were aspirated and DMSO (solubilisation solution; 200 µL/well) was added. The DMSO was then pipetted up and down twice per well in order to obtain a uniform purple colour in each well. The absorbance value of each well was measured at 550 nm using a BioTek® PowerWave 340 spectrophotometer (Biotek® instruments, USA). Results were expressed as fold induction relative to the negative control (0.1% (v/v) DMSO) for agonism or relative to 10^{-9}M E_2 for antagonism, which was set as one. For investigation in agonist mode test samples were administered alone, whereas for investigation in antagonist mode, test samples were administered in the presence of 10^{-9}M E_2.

3.2.3 Characterisation of phenolic content

3.2.3.1 Determination of total polyphenol content

The method was adapted from Singleton and Rossi [70] to accommodate 96-well tissue culture plates (Greiner Bio-One®). Initially, 100 µl 10% (v/v) Folin-Ciocalteau reagent (Merck®) was added to 20 µl of sample (0.5 mg/ml DME) and gallic acid (Sigma Aldrich®) standards (0.01-0.1mg/ml), followed by the addition of 80 µl (7.5%) Na_2CO_3 (Sigma Aldrich®). The 96-well plates were then incubated at 37°C for 2 h, where after absorbance was measured at 765 nm using a BioTek® PowerWave 340 spectrophotometer. Extract gallic acid equivalents (GAE) were obtained from the standard curve and the total polyphenol content, determined in triplicate, expressed as g GAE/100 g of extract.

3.2.3.2 Determination of total flavanoid content

The method, described by Zishen *et al.* [71], was adapted to 96-well tissue culture plate format. Initially, 6 µl 5% (w/v) $NaNO_2$ (Sigma Aldrich®) was added to 100 µl of standard (rutin hydrate (0.01-0.1 mg/ml) from Sigma Aldrich®) and DME (0.05 mg/ml). The reaction was allowed to proceed for 5 min before 60 µl 10% (w/v) $AlCl_3$ (Merck®) was added. The reaction was allowed to proceed for a further 6 min before adding 34 µl NaOH (1M) (Saarchem®) followed by incubation at room temperature for 20 min. The 96-well plates were then incubated at 37°C for 2 h, where after

absorbance was measured at 450 nm using a BioTek® PowerWave 340 spectrophotometer. Extract rutin hydrate equivalents (RE) were obtained from the standard curve. The total flavonoid content, determined in triplicate, was expressed as g RE/100 g of extract.

3.2.3.3 Quantification of phenolic compounds using HPLC-DAD

Stock solutions of standards and SM6Met, as well as liquid-liquid and HPCCC fractions, were prepared in DMSO and frozen at -20°C until needed for analysis. For experimental analysis, the defrosted extracts, fractions and standards were appropriately diluted with water and ascorbic acid (Sigma Aldrich®) was added to a final concentration of 9 mg/mL. The mixtures were then filtered using Millex-HV® syringe filters (Millipore®, USA) with a 0.22 µm pore size. Separation was achieved on a Gemini-NX C18® column (150 × 4.6 mm; 3 µm; 110 Å; Phenomenex®, Santa Clara, U.S.A.), protected by a guard column (4 x 3.0 mm; Phenomenex®) of the same stationary phase , with 2% acetic acid (A) and HPLC-grade acetonitrile (B) as mobile phases. Injection volumes ranged from 10-20 µL for standards and 15 µL for the extracts and fractions. Separation was performed using the HPLC method described by De Beer *et al.* [72]. A flow rate of 1 mL/min was used with the following mobile phase gradient: 0-2 min (8% B), 2-27 min (8-38% B), 27-28 min (38-50% B), 28-29 min (50% B), 29-30 min (50-8% B), 30-40 min (8% B).

The dihydrochalcones (phloretin-3',5'-di-*C*-β-glucoside and 3-hydroxyphloretin-3',5'-di-*C*-hexoside), flavanones (eriocitrin and hesperidin), benzophenone (iriflophenone-3-*C*-glucoside) and protocatechuic acid were quantified at 288 nm, whereas the xanthones (mangiferin and isomangiferin), flavones (luteolin and scolymoside) and *p*-coumaric acid were quantified at 320 nm. A 7-point calibration curve was set up for all the available standards, including standards needed to calculate equivalent values to accommodate the following compounds in the extracts and fractions: luteolin (Extrasynthese®), mangiferin (Sigma-Aldrich®), isomangiferin (Chemos GmbH®), eriocitrin (Extrasynthese®), hesperidin (Sigma-Aldrich®), aspalathin (kind gift from Prof. W. Gelderblom, PROMEC unit, Medical Research Council, Tygerberg, South Africa), protocatechuic

acid and *p*-coumaric acid (Fluka™ Analytical, Sigma-Aldrich®), apigenin (Fluka™ Analytical, Sigma-Aldrich®), and nothofagin (kind gift from Prof. W. Gelderblom). Iriflophenone-3-*C*-glucoside and iriflophenone-di-*O,C*-hexoside were quantified using iriflophenone-3-*C*-glucoside isolated from *C. genistoides* (kind gift from Dr. E. Willenburg, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). Scolymoside and vicenin-2 were expressed as luteolin and apigenin equivalents, respectively, as no authentic reference standards were available for these compounds. Phloretin-3',5'-di-*C*-β-glucoside and 3-hydroxyphloretin-3',5'-di-*C*-hexoside where expressed in terms of nothofagin (phloretin-3'-*C*-β-glucoside) and aspalathin (3-hydroxyphloretin-3'-*C*-β-glucoside) equivalents, respectively.

3.2.3.4 Identification of phenolic compounds using LC-MS/MS analysis

Analyses were performed on a WatersAcquity® ultra-performance liquid chromatography (UPLC) system comprising an in-line degasser, diode array detector (DAD), column oven and binary pump. This system was coupled to a Synapt G2® quadrupole time of flight (QTOF) mass spectrometer (Waters®) containing an electrospray ionization (ESI) source. The method used was the same as described in De Beer *et al.* [72] for LC-MS/MS analysis. An injection volume of 10 µL was used for samples with the eluent being split 1:1 before introduction to the ionization source. MS results were acquired in the negative ionization mode with MS and MS^E for each sample. For MS^E a collision energy ramp from 25 to 60 V was used, whereas for MS/MS data a collision energy of 30 V was used. The parameters used for MS as in De Beer *et al*. [72] were as follows: capillary voltage, 2.5 kV, cone voltage 15 V, desolvation temperature 275°C, source temperature 120°C and nitrogen flow rate 650 L/h. Peaks were identified by comparing LC-MS spectra, UV-Vis spectra and retention times to authentic standards or as in De Beer *et al*. [72]. Data were acquired and processed by means of MassLynx v4.1® software (Waters®).

3.2.4 Data manipulation and statistical analysis

GraphPad Prism® version 5 (GraphPad Software®, USA) was used for graphical representation and statistical analysis of experimental data. One-way analysis of variance (ANOVA) was performed with Dunnett's multiple comparisons test as post-test. For all experiments, unless otherwise indicated, the error bars represent the SEM (standard error of means) of three independent experiments done in triplicate.

3.3 Results

We used the positive estrogenic attributes previously identified [62-64] as a guide during activity-guided fractionation of SM6Met. Specifically, ERα antagonism and ERβ agonism were evaluated using an ERE-containing promoter reporter in the HEK293 cell line. The HEK293 cell line is ideally suited for this purpose as steroid receptor characterisation using Western blotting (Fig. S7) indicated that this cell line contains only GR and MR, AR, or either of the PR isoforms, PRA or PRB. More importantly, the HEK293 cells do not contain endogenous ERα or ERβ (Fig. S7), which was confirmed by performing a whole cell binding assay using tritiated E_2 (Fig. S8). Thus, HEK293 cells could be transfected with expression vectors for ERα or ERβ, which allowed for investigation of ER-subtype specific responses. Furthermore, the HEK293 cell line responds well to E_2-treatment, which served as a positive control throughout the promoter reporter studies [65]. In addition, antagonism of E_2-induced breast cancer cell proliferation was investigated in MCF-7BUS cells. The use of this model is merited by the endogenous presence of both ERα and ERβ, as demonstrated by Western blotting (Fig. S9). Furthermore, it is a more complex model representing both activation and repression of genes, and it provides a physiologically relevant system, as breast cancer is a topic of interest in the development of a phytoestrogenic nutraceutical from *Cyclopia*. In addition, LC-MS and qHPLC were used to track major polyphenols during fractionation to allow for correlation with estrogenic activity in an attempt to identify possible marker compounds.

3.3.1 Activity-guided fractionation of SM6Met

To assist the reader we provide an overview of the fractionation process and yields obtained (Fig 1). Initially, SM6Met was "mass-produced" by several repeats (n = 4) of the process described by Mfenyana *et al.* [63]. SM6Met was subsequently fractionated into polar (PF) and NPF using liquid-liquid fractionation and the NPF was sub-fractionated via HPCCC yielding F1-F3.

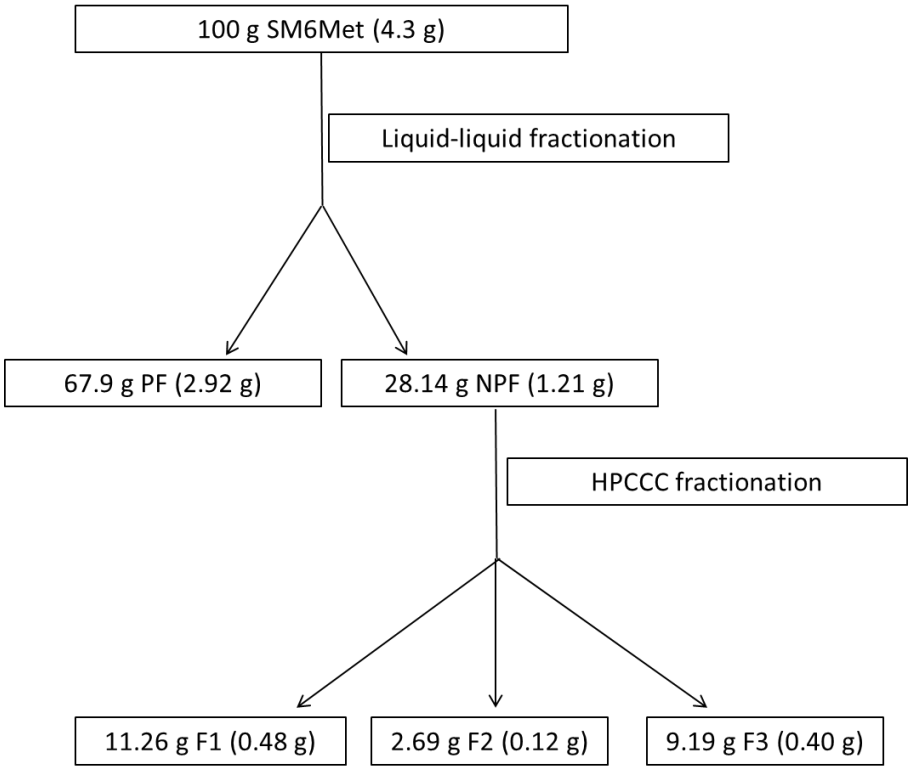

Figure 1. Overview of extract yield during liquid-liquid and HPCCC fractionation. Yields are expressed as if 100 g SM6Met was used in fractionation. Values in brackets represent the actual mass used during this study as 4.3 g SM6Met was subjected to liquid-liquid fractionation. However, only 450 mg NPF was used to produce 180 mg F1, 43 mg F2, and 147 mg F3, thus in the diagram these values were recalculated as if 1.21g NPF was used.

3.3.1.1 Large scale production and evaluation of SM6Met

SM6Met was prepared (Fig. S1) as in Mfenyana *et.al* [63] in 4 separate batches (B1-B4), mainly to check for inter batch variability in the production of the extract. A small quantity of each individual batch was retained for assays and the remainder pooled to form the B1-4Mix. Both individual batches and the B1-4Mix were compared to a reference SM6Met sample produced by Mfenyana *et al.* [63].

Comparison of extract yields (g extract/100 g dried plant material) indicated a yield ranging from 13.0% for B1 to between 16.1 and 16.6% for B2 to B4 (Table 1). In terms of comparison with the reference sample (Mfenyana) the yields of the individual batches were quite similar to that of the reference batch.

Although Mfenyana *et al*. [63] indicated that no correlation existed between estrogenic activity of extracts and their total polyphenol content (TPC) or total flavonoid content (TFC), we decided to use these two parameters as an additional measure of comparison between the reference (Mfenyana) and newly prepared batches. The TPC and TFC of the newly produced SM6Met batches (B1 to B4) ranged from 40.72-44.34 g GAE/100 g extract and 47.62-73.99 g RE/100g extract, respectively (Table 1). There was no significant difference between the TPC and TFC values of the newly produced batches. Comparison of the B1-4Mix to the Mfenyana reference sample also showed no significant difference for TPC and TFC.

Table 1: Inter batch variability of SM6Met batches. Extracts were produced in four batches B1-B4 and were then pooled to form B1-4Mix. Inter batch variability is shown by means of yield, total polyphenol content (TPC) and total flavonoid content (TFC). B1-B4 were prepared as in Mfenyana *et al*. [63] and compared to the original SM6Met produced by Mfenyana *et al*. [63]. The table shows results of two independent experiments for TPC and TFC. For statistical analysis One-way ANOVA was used with Dunnett's Multiple Comparison Test as post-test. No symbol indicates no significant differences compared to Mfenyana *et al*. and also compared to B1-4Mix.

SM6Met extract	Yield[a]	TPC[b]	TFC[c]
Mfenyana	15.4	43.09 ± 11.11	69.27 ± 6.820
B1	13.0	41.00 ± 11.52	48.26 ± 11.18
B2	16.1	42.45 ± 12.02	73.99 ± 2.807
B3	16.5	40.72 ± 9.56	60.62 ± 9.147
B4	16.6	44.34 ± 12.94	47.62 ± 4.491
B1-4Mix	-	43.01 ± 12.31	62.46 ± 12.26

[a] g/100 g plant material
[b] g gallic acid equivalents (GAE)/100 g extract
[c] g rutin hydrate equivalents (RE)/100 g extract

Although SM6Met has been shown to display ERα antagonism and ERβ agonism in COS-1 cells [64], we for the first time investigated estrogenic activity of a *Cyclopia* extract in HEK293 cells. In our model we transfected HEK293 cells with either ERα or ERβ and an ERE-containing promoter reporter construct (ERE.vit2.luc) and investigated transactivation in both agonist and antagonist mode (Fig. 2). We found that 1 nM of E_2 induced a 3.1 fold and 6.3 fold induction via ERα and ERβ, respectively. Although, this induction level is lower than previously found in HEK293 cells [65], it is a significant improvement of induction levels found in COS-1 cells [64].

All our newly prepared SM6Met batches, with the exception of B1, showed significant ($p < 0.01$) ERα antagonism (Fig. 2C), one of the positive estrogenic attributes of interest. Despite the fact that B1 did not display ERα antagonism, the mix of batches, SM6Met B1-4Mix, retained the desired ERα antagonist activity. Furthermore, the ERα antagonist activity of this mix was not significantly different from the reference sample from Mfenyana. Despite the fact that our newly prepared extracts displayed ERα antagonism, as previously demonstrated in COS-1 cells [64], we now, in contrast to previous results, also showed significant agonism via ERα, not only for the reference sample from Mfenyana, but also for the newly produced B1 and SM6Met B1-4Mix (Fig. 2A). This conflict with previous results may be explained by the fact that the HEK293 cells displayed a greater estrogenic sensitivity than the COS-1 cells, as demonstrated by the differences in response to E_2. Extract-induced ERα agonism was, however, significantly lower ($p < 0.001$) than that of 10^{-11}M E_2 for all batches except B1 (Fig. 2A).

The second desired positive estrogenic attribute we investigated was ERβ agonism. Of the newly prepared batches B1, B4 and SM6Met B1-4Mix all showed significant ($p < 0.05$) ERβ agonism (Fig. 2B), which in the case of B1 and SM6Met B1-4Mix was significantly ($p < 0.05$) higher than 10^{-11}M E_2. These results suggest that SM6Met B1-4Mix is a stronger ERβ agonist than an ERα agonist. Furthermore, SM6Met B1-4Mix displayed ERβ agonism that was not significantly ($p > 0.05$) different from that of the reference Mfenyana sample. Neither of the newly prepared SM6Met

batches, except B4, nor the reference Mfenyana sample displayed ERβ antagonism (Fig. 2D).

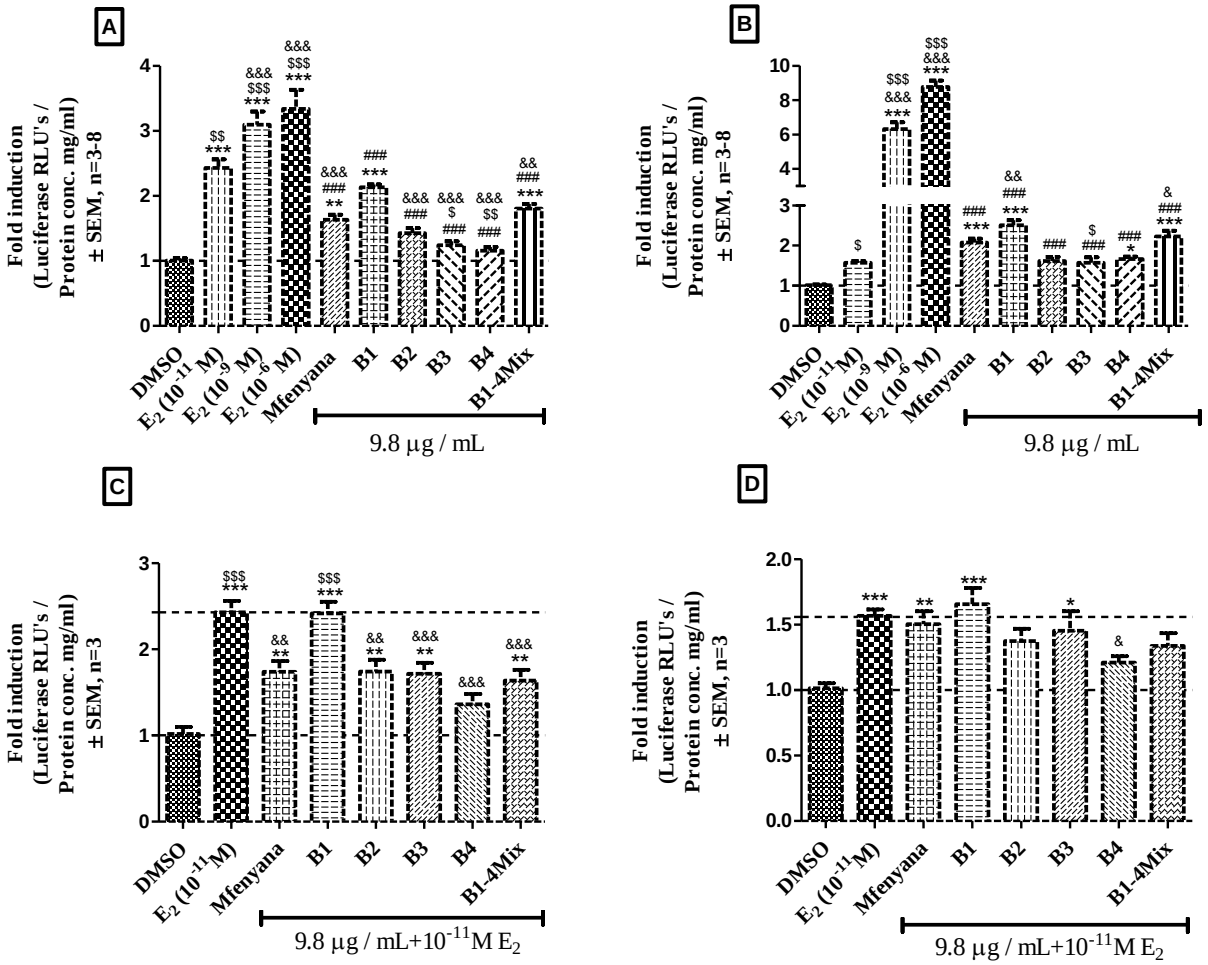

Figure 2. Transactivation studies investigating 17β-estradiol (E_2) and SM6Met produced: Mfenyana *et al.* [63]**, batches B1-4 and B1-4Mix. A-B agonist mode; C-D antagonist mode** (A) HEK293 cells transfected with pSG5-hERα, ERE.vit2.luc and pGL2-Basic were induced with 10^{-11}-10^{-6} M E_2, 9.8 µg/mL SM6Met from Mfenyana *et al.* [63], B1-B4, or B1-4Mix. (B) HEK293 cells transfected with pSG5-hERβ, ERE.vit2.luc and pGL2-Basic were induced as in A. (C) HEK293 cells were transfected as in A and induced with 10^{-11} M E_2, 9.8 µg/mL SM6Met from Mfenyana *et al.* [63], B1-B4, or B1-4 Mix, with the extract treatments co-administered with10^{-11}M E_2. (D) HEK293 cells were transfected as in B and induced with 10^{-11} M E_2, 9.8 µg/mL SM6Met from Mfenyana *et al.* [63], B1-B4, or B1-4 Mix, with the extract treatments co-administered with10^{-11} M E_2. For statistical analysis one-way ANOVA was used with Dunnett's Multiple Comparisons Test as post-test. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$. For all bars compared to 1nM E_2 ### denotes $p < 0.001$, ## denotes $p < 0.01$ and # denotes $p < 0.05$. For all bars compared to B1-4Mix $$$ denotes $p < 0.001$, $$ denotes $p < 0.01$ and $ denotes $p < 0.05$. For all bars compared to 10^{-11}M E_2 &&& denotes $p < 0.001$, && denotes $p < 0.01$ and & denotes $p < 0.05$. No symbol above bars indicates no significant differences compared to the above mentioned compared conditions.

The third and final positive estrogenic attribute investigated was antagonism of E_2-induced breast cancer cell proliferation in MCF-7BUS cells. The MTT assay was used to evaluate the number of viable cells, however, as it has been shown that some polyphenols may influence the MTT assay

without affecting cell viability [73], we conducted an experiment in which we compared proliferation results obtained from an MTT assay with results obtained from cell counting (data not shown). As the results obtained were similar we decided to use the MTT assay for further analyses. Although only one of the newly prepared SM6Met batches, B4, displayed significant antagonism, the SM6Met B1-4Mix showed significant ($p < 0.01$) antagonism (Fig. 3B). Furthermore, this antagonism of SM6Met B1-4Mix was not significantly different from the reference Mfenyana sample. Despite displaying antagonism of E_2-induced breast cancer cell proliferation, the reference Mfenyana sample also induced proliferation in the agonist mode (Fig. 3A), however, to a significantly ($p < 0.05$) lesser extent than 1nM E_2. None of the newly prepared SM6Met batches, except B1, displayed induction of breast cancer cell proliferation in agonist mode (Fig. 3A).

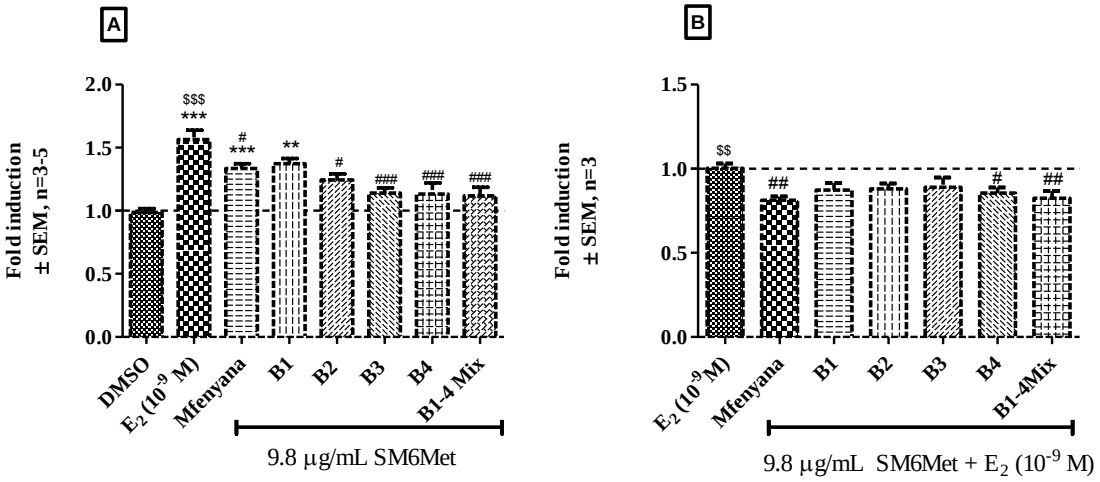

Figure 3. Proliferation studies investigating E_2 and SM6Met produced: Mfenyana *et al.* [63]**, batches B1-4 and B1-4Mix. A-B agonist mode; C-D antagonist mode.** MCF-7 BUS cells were serum starved for 24 h, where after cells were induced at 24 h, and re-induced at 48 h and 72 h. The assay was performed at 96 h. In (A) cells were induced with 10^{-9} M E_2, 9.8 µg/mL SM6Met from Mfenyana *et al*. [63], B1-B4, or B1-4 Mix. In (B) MCF-7 BUS cells were treated as in (A) with 9.8 µg/mL SM6Met from Mfenyana *et al*. [63], B1-B4, or B1-4Mix, with the extract treatments co-administered with10^{-9} M E_2. For statistical analysis one-way ANOVA was used with Dunnett's Multiple Comparison Test as post-test. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$. For all bars compared to 1 nM E_2 ### denotes $p < 0.001$, ## denotes $p < 0.01$ and # denotes $p < 0.05$. For all bars compared to B1-4Mix $$$ denotes $p < 0.001$, $$ denotes $p < 0.01$ and $ denotes $p < 0.05$. No symbol above bars indicates no significant differences compared to the above mentioned compared conditions.

Having investigated the positive estrogenic attributes of the newly prepared batches (B1-B4) as well as SM6Met B1-4Mix, we were interested in whether the polyphenolic compounds correlated with the reference Mfenyana sample and wanted to establish a baseline polyphenol profile of SM6Met

B1-4Mix to which subsequent fractions, which would be prepared from this mix, could be compared. LC-MS/MS analysis was used to identify compounds present in SM6Met B1-4Mix (Table S3, Fig. S10A). Most of the identified compounds had previously been identified in aqueous *Cyclopia subternata* extracts prepared from a large number of seedling plants [72]. Some new compounds were, however, tentatively identified namely, luteolin-*O*-hexoside, apigenin-*O*-rutinoside /neohesperidoside (flavone), chrysoeriol-*O*-rutinoside/neohesperidoside (flavone), and quercetin-*O*-rutinoside/neohesperidoside (flavonol). These newly identified compounds were, however, present at low concentrations, and may be considered minor compounds. qHPLC was used to quantify the major phenolic compounds, i.e. iriflophenone-3-*C*-glucoside, mangiferin, isomangiferin, scolymoside, hesperidin, eriocitrin, phloretin-3',5'-di-*C*-glucoside and 3-hydroxyphloretin-3',5'-di-*C*-hexoside, as well as the phenolic acids, *p*-coumaric acid and protocatechuic acid (Table 2, Fig. S11A). In addition, luteolin was also quantified as previous studies had shown that the presence of luteolin may correlate with estrogenic activity of extracts [62,63,74]. When we calculated the percentage of major quantified compounds, the flavanones, hesperidin and eriocitrin, and the xanthones, mangiferin and isomangiferin, were present at the highest percentage. Specifically, the flavanones constituted 30.4%, and the xanthones 26.7% of the quantified major phenolic compounds (Table 2). The dihydrochalcones, 3-hydroxyphloretin-3',5'-di-*C*-hexoside and phloretin-3',5'-di-*C*-glucoside, were present at 20.7%, while the benzophenone, iriflophenone-3-*C*-glucoside, comprised 7% of the quantified major phenolic compounds. The flavones, luteolin and its rutinoside, scolymoside, were present at 13.9%, while the phenolic acids, *p*-coumaric acid and protocatechuic acid, were only present in trace amounts. Comparison of SM6Met B1-4Mix with literature values previously obtained for the reference sample from Mfenyana [63] indicated that SM6Met B1-4Mix contained similar amounts of phloretin-3',5'-di-*C*-glucoside and the xanthones, flavones and flavanones (Table 2). *p*-Coumaric acid, protocatechuic acid, iriflophenone-3-*C*-glucoside and 3-hydroxyphloretin-3',5'-di-*C*-hexoside, were not previously

determined and thus we cannot compare their concentrations in the current B1-4Mix to the reference sample from Mfenyana.

In terms of identifying polyphenol marker compounds contributing to the estrogenic activity of the SM6Met B1-4Mix, luteolin, iriflophenone-3-*C*-glucoside and eriocitrin may contribute as they have previously been shown to have estrogenic activity [61,62,74-78], although in the case of iriflophenone-3-*C*-glucoside, only the aglycone, iriflophenone, was investigated. Mangiferin, hesperidin and *p*-coumaric acid would be unlikely to contribute to the estrogenic activity of the extract as previous studies have indicated that they lack estrogenic activity [61,62,78-80]. The other compounds have not been tested for estrogenic activity and thus we cannot yet predict a correlation with activity.

Table 2: Polyphenol content of SM6Met B1-4Mix as determined by qHPLC and compared to Mfenyana *et al.* [63]

Polyphenol	B1-4Mix g/100 g extract	B1-4Mix % polyphenol present[d]	Mfenyana *et al.* (g/100 g extract)
Mangiferin	1.899	19.9	1.85
Isomangiferin	0.645	6.8	0.75
Scolymoside (luteolin-7-*O*-rutinoside)[a]	1.289	13.5	1.82
Luteolin	0.040	0.4	0.04
Iriflophenone-3-*C*-glucoside	0.669	7.0	na[e]
Phloretin-3',5'-di-*C*-glucoside[b]	1.278	13.4	1.27
3-Hydroxyphloretin-3',5'-di-*C*-hexoside	0.700	7.3	na
Eriocitin	0.845	8.9	1.25
Hesperidin (hesperetin-7-*O*-rutinoside)	2.049	21.5	1.87
Protocatechuic acid	0.113	1.2	na
***p*-Coumaric acid**	co-elution[c]		na

[a] previously described as unknown 1
[b] previously described as unknown 2
[c] Co-elution = p-coumaric acid co-elutes with 3-hydroxyphloretin-3',5'-di-*C*-hexoside where present; this means that the concentration could not be determined accurately if 3-hydroxyphloretin-3',5'-di-*C*-hexoside was present.
[d] % of polyphenol present calculated relative to total concentration of major polyphenols in extract
[e] na = not assayed

Thus in summary, the newly prepared SM6Met B1-4Mix retained the positive estrogenic attributes previously described [63,64], namely, ERα antagonism, ERβ agonism and antagonism of E_2-induced breast cancer cell proliferation. However, in addition some weak ERα agonism was observed. SM6Met B1-4Mix, like the reference Mfenyana sample, contained mostly xanthones and

flavanones, and also significant amounts of dihydrochalcones, flavones and benzophenones, of which mostly the flavanones and flavones had previously been shown to display estrogenic activity. There was some heterogeneity in the estrogenic activity of the individual B1-B4 batches and in hindsight we should have tested these batches before mixing, specifically, batch B1 could have been excluded.

3.3.1.2 Liquid-liquid fractionation of SM6Met B1-4Mix to obtain a polar fraction and a non-polar fraction

SM6Met B1-4Mix was fractionated into the two fractions, PF and NPF, according to relative polarity by means of liquid-liquid fractionation using water to dissolve/suspend the extract and n-BuOH as less polar solvent. Liquid-liquid fractionation of 4.3 g SM6Met resulted in 1.21 g NPF and 2.92 g PF (Fig.1), giving a total recovery of 96%. qHPLC analysis was performed to characterise their individual phenolic compound content. The resultant PF and NPF were then evaluated for estrogenic potential using the promoter reporter and cell proliferation assays.

Both PF and NPF retained significant ($p < 0.05$) ERα antagonist activity, one of the positive estrogenic attributes of interest. In addition, in contrast to the batch of origin, SM6Met B1-4 Mix, which displayed significant ($p < 0.01$) ERα activation (Fig. 4A), neither PF nor NPF showed activation via ERα. (Fig. 4A). The absence of ERα agonism, seen for PF and NPF, positions them more favourably in terms of positive estrogenic attributes compared to the original SM6Met B1-4Mix as ERα agonism is known to be pro-proliferative [44].

ERβ agonism, another positive estrogenic attribute of interest, was retained by NPF, which showed significant ($p > 0.05$) ERβ agonism, as seen for the extract of origin, SM6Met B1-4Mix (Fig. 3B). PF, however, displayed no significant ERβ agonism. In addition, no ERβ antagonism was seen for either fraction (Fig. 4D). The additive effect seen for NPF in antagonist mode supports the evidence that this fraction is an ERβ agonist. Furthermore, the fact that for NPF a significantly ($p < 0.01$)

higher ERβ activation response in antagonist mode was observed when compared to SM6Met B1-4Mix, suggests that the NPF is a stronger ERβ agonist than SM6Met B1-4Mix.

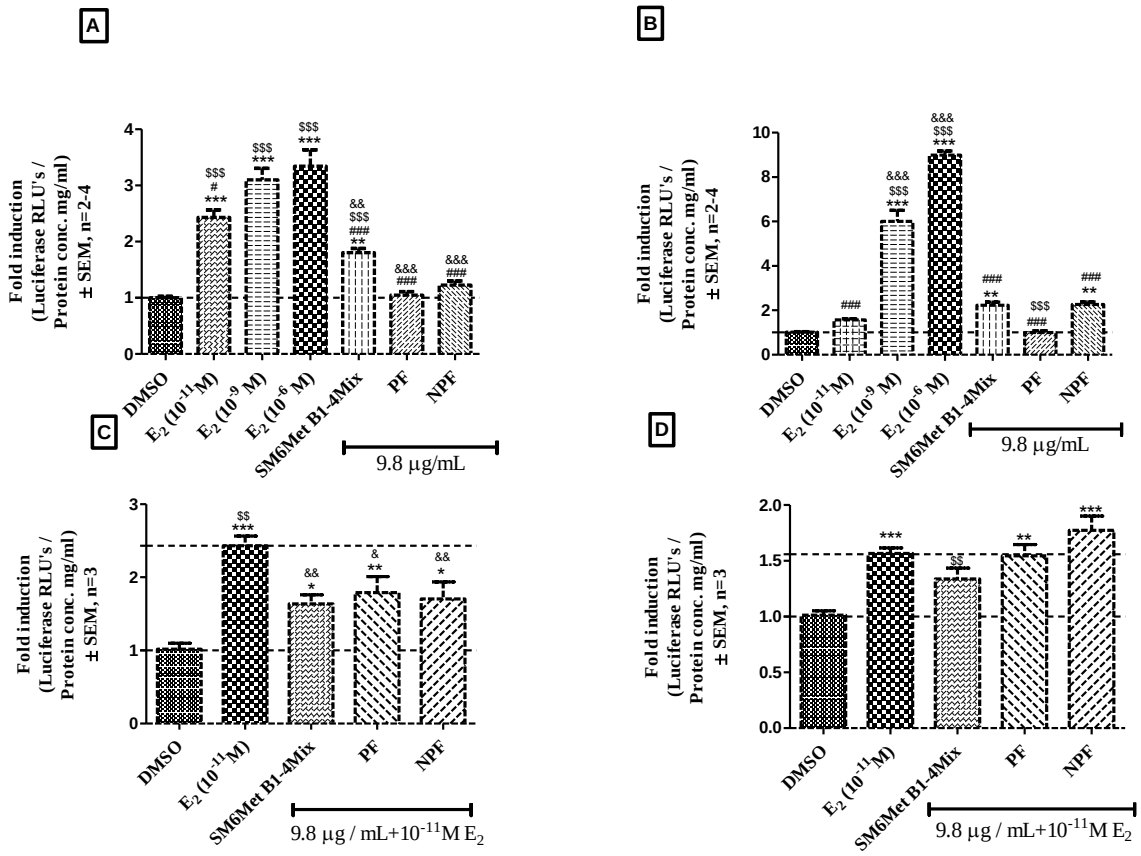

Figure 4. Transactivation studies investigating E_2, SM6Met B1-4Mix and liquid-liquid fractionation produced NPF and PF: A-B agonist mode; C-D antagonist mode (A) HEK293 cells transfected with pSG5-hERα, ERE.vit2.luc and pGL2-Basic were induced with 10^{-6}-10^{-11}M E_2, 9.8 µg/mL SM6Met B1-4Mix, PF or NPF. (B) HEK293 cells transfected with pSG5-hERβ, ERE.vit2.luc and pGL2-Basic were induced as in A. (C) HEK293 cells were transfected as in A induced with 10^{-11} M E_2, 9.8 µg / mL SM6Met B1-4Mix, PF or NPF, with the sample treatments co-administered with10^{-11} M E_2. (D) HEK293 cells were transfected as in B and induced with 10^{-11} M E_2, 9.8 µg/mL SM6Met B1-4Mix, PF or NPF, with the sample treatments co-administered with 10^{-11} M E_2. For statistical analysis one-way ANOVA was used with Dunnett's Multiple Comparisons Test as post-test. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$. For all bars compared to 1 nM E_2 ### denotes $p < 0.001$, ## denotes $p < 0.01$ and # denotes $p < 0.05$. For all bars compared to NPF $$$ denotes $p < 0.001$, $$ denotes $p < 0.01$ and $ denotes $p < 0.05$. For all bars compared to 10^{-11} M E_2 &&& denotes $p < 0.001$, && denotes $p < 0.01$ and & denotes $p < 0.05$. No symbol above bars indicates no significant differences compared to the above mentioned compared conditions.

In accordance with SM6Met B1-4Mix activity, significant ($p < 0.01$) antagonism of E_2-induced breast cancer cell proliferation, the final positive estrogenic attribute investigated, was seen for both PF and NPF (Fig. 5B). In addition, like SM6Met B1-4Mix, PF and NPF did not induce significant ($p > 0.05$) breast cancer cell proliferation (Fig. 5A).

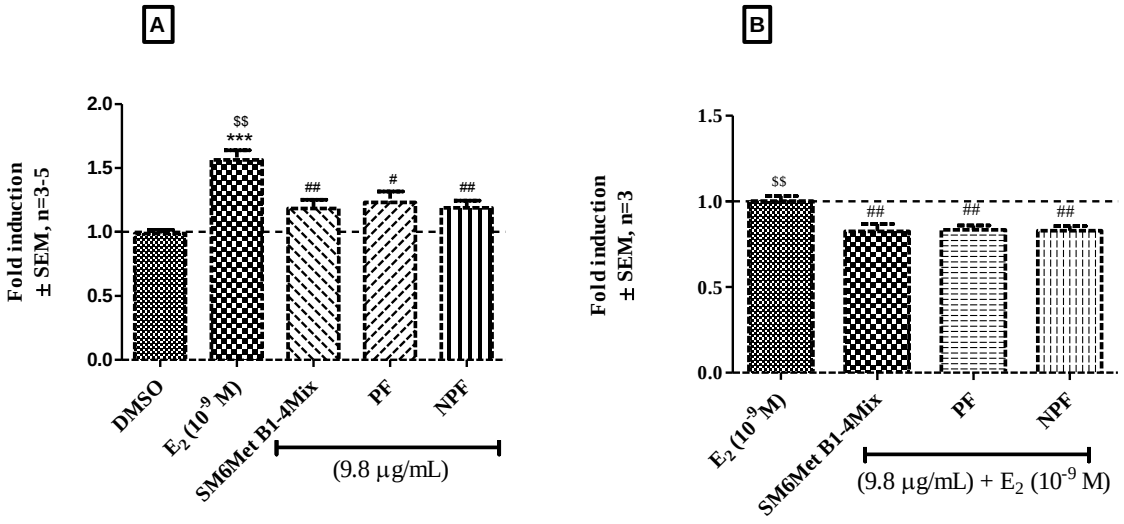

Figure 5. Proliferation studies investigating E_2, SM6Met B1-4Mix and liquid-liquid fractionation produced NPF and PF: A agonist mode; B antagonist mode. MCF-7 BUS cells were serum starved for 24 h, where after cells were induced at 24 h, and re-induced at 48 h and 72 h. The assay was performed at 96 h. In (A) cells were induced with 10^{-9} M E_2, 9.8 µg/mL SM6Met B1-4Mix, PF or NPF. In (B) MCF-7 BUS cells were treated as in (A) with 10^{-9} M E_2, 9.8 µg/mL SM6Met B1-4 Mix, PF or NPF, with the test sample treatments co-administered with10^{-9} M E_2. For statistical analysis one-way ANOVA was used with Dunnett's Multiple Comparisons Test as post-test. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$. For all bars compared to 1 nM E_2 ### denotes $p < 0.001$, ## denotes $p < 0.01$ and # denotes $p < 0.05$. For all bars compared to NPF $$$ denotes $p < 0.001$, $$ denotes $p < 0.01$ and $ denotes $p < 0.05$. No symbol above bars indicates no significant differences compared to the above mentioned compared conditions.

In an attempt to link positive estrogenic effects of PF and NPF to polyphenolic constituents in the fractions, LC-MS/MS analysis (Table S3, Figs. S10B&C) was performed to track major and minor compounds during the liquid-liquid fractionation procedure. Results (Table S3, Figs. S10B&C) showed that all of the major compounds in SM6Met B1-4Mix were retained as major compounds in NPF, whereas for PF most of these major compounds, with the exception of 3-hydroxyphloretin-3',5'-di-*C*-hexoside, eriocitrin, scolymoside and phloretin-3',5'-di-*C*-glucoside, were now present as minor compounds. In addition, qHPLC analysis of the major compounds in NPF and PF showed an overall 2 to 3-fold increase for NPF (Table 3, Figs. S11B&C), and a 3 to 20 fold decrease for PF compared to the SM6Met B1-4Mix. Analysis of NPF showed that the xanthones, flavones, flavanones and dihydrochalcones each contributed 14.8-29.01% towards the quantified phenolic compounds, whereas the benzophenone, iriflophenone-3-*C*-glucoside, and the phenolic acid, protocatechuic acid, contributed 6.07 and 1.35%, respectively, towards the quantified phenolic compounds. Thus, as a percentage of the major phenolic compounds the xanthones, flavones, and

protocatechuic acid increased, while iriflophenone-3-*C*-glucoside, flavanones, and dihydrochalcones decreased in NPF relative to the source SM6Met B1-4Mix. In contrast, although in much lower quantities, PF consisted mostly of flavanones (36.64%), followed by the dihydrochalcones, 3-hydroxyphloretin-3',5'-di-*C*-hexoside, and phloretin-3',5'-di-*C*-glucoside (31.61%), the benzophenone, iriflophenone-3-*C*-glucoside (15.21%), and low amounts of xanthones (9.4%), and the flavone, scolymoside (7.14%).

Table 3: Individual polyphenol content as determined by qHPLC, concentration change relative to SM6Met B1-4Mix, and % polyphenol present as per total quantified polyphenols present in NPF and PF.

	g/100 g extract		Fold change relative to SM6Met B1-4Mix[a]		% polyphenol present[b]	
Polyphenol	**PF**	**NPF**	**PF**	**NPF**	**PF**	**NPF**
Mangiferin	0.094	5.559	0.05	2.93	6.22	21.55
Isomangiferin	0.048	1.879	0.08	2.91	3.18	7.29
Scolymoside (luteolin-7-*O*-rutinoside)	0.111	3.710	0.08	2.88	7.14	14.38
Luteolin	Nd[c]	0.105	Nd	2.64	Nd	0.41
Iriflophenone-3-*C*-glucoside	0.230	1.565	0.34	2.34	15.21	6.07
Phloretin-3',5'-di-*C*-glucoside	0.251	3.456	0.20	2.70	16.60	13.4
3-Hydroxyphloretin-3',5'-di-*C*-hexoside	0.227	1.686	0.32	2.41	15.01	6.54
Eriocitrin (eriodictyol-7-*O*-rutinoside)	0.187	2.129	0.22	2.52	12.37	8.25
Hesperidin (hesperetin-7-*O*-rutinoside)	0.367	5.356	0.18	2.61	24.27	20.76
Protocatechuic acid	Nd	0.347	Nd	3.07	Nd	1.35
***p*-Coumaric acid**	Nd	NA[d]	Nd	NA	Nd	NA

[a] Polyphenol concentration (g/100g extract) present in fraction relative to polyphenols in SM6Met B1-4Mix (Table 2), set as 1
[b] % of polyphenol present calculated relative to total content of major polyphenols in fraction
[c] Nd - not detected due to absence or very low amounts
[d] NA - value not available as *p*-coumaric acid co-eluted with 3-hydroxyphloretin-3',5'-di-*C*-hexoside where present

In terms of identifying polyphenol marker compounds contributing to the estrogenic activity of NPF and PF it is clear that, amongst the major polyphenolic compounds (Table 3), the compounds identified as possibly contributing to the positive estrogenic attributes, luteolin, iriflophenone-3-*C*-glucoside and eriocitrin are all retained and concentrated in NPF, in contrast to PF where only iriflophenone-3-*C*-glucoside and eriocitrin were retained, but not luteolin. These two compounds were present, however, at much lower concentrations in PF than in SM6Met B1-4Mix (Table 3).

Thus in summary, NPF retained all of the positive estrogenic attributes previously described for SM6Met B1-4Mix, namely, ERα antagonism, ERβ agonism and antagonism of E_2-induced breast cancer cell proliferation, whereas PF retained only ERα antagonism, and antagonism of E_2-induced breast cancer cell proliferation, but not ERβ agonism. Furthermore, the weak ERα agonism observed for SM6Met B1-4Mix was lost during fractionation into PF and NPF. Interestingly, despite the fact that all of the quantified major phenolic compounds in the batch of origin, SM6Met B1-4Mix, increased in concentration in NPF we did not see a significant increase in either ERα antagonism nor ERβ agonism, while antagonism of E_2-induced breast cancer cell proliferation also remained constant. As ERβ agonism is considered an important positive estrogenic attribute for the development of a possible phytoestrogenic nutraceutical, due to its anti-proliferative function [44], we decided to focus on NPF, rather than PF, for subsequent fractionation.

3.3.1.3 Fractionation of the non-polar fraction by high performance counter-current chromatography to obtain F1-F3

As NPF retained the positive estrogenic attributes of interest, namely, ERα antagonism, ERβ agonism and antagonism of E_2-induced breast cancer cell proliferation, we decided to fractionate it further using HPCCC. Consequently, HPCCC fractionation of NPF yielded three major fractions (F1, F2 and F3) (Fig. 1), with 0.45 g NPF resulting in 0.180 g F1, 0.043 g F2 and 0.147 g F3. The overall yield of these three HPCCC fractions was 82.2%. For evaluation of the fractions for desired estrogenic attributes and to track polyphenolic compounds for correlation with activity, F1-F3 were subjected to the same two estrogenicity assays previously used and analysed by qHPLC and LC-MS/MS.

In accordance with the activity of NPF, F1 and F2 displayed significant (p <0.001) ERα antagonism, a desirable estrogenic trait. F3 displayed no ERα antagonism in the presence of E_2 as it induced activity not significantly (p > 0.05) different from that of E_2 alone (Fig. 6C). In addition, none of the fractions, with the exception of F3, displayed significant ERα agonism (Fig. 6A).

However, the ERα agonism of F3 was weak and significantly ($p < 0.001$) lower than that of 10^{-11} M E_2, reflecting an "activity profile" quite similar to that of the original SM6Met B1-4Mix (Fig. 2A). This change in activity may be linked to the increased presence of polyphenolic compounds inducing ERα agonism, or it may be due to the decrease or loss of polyphenolic compounds linked to ERα antagonism.

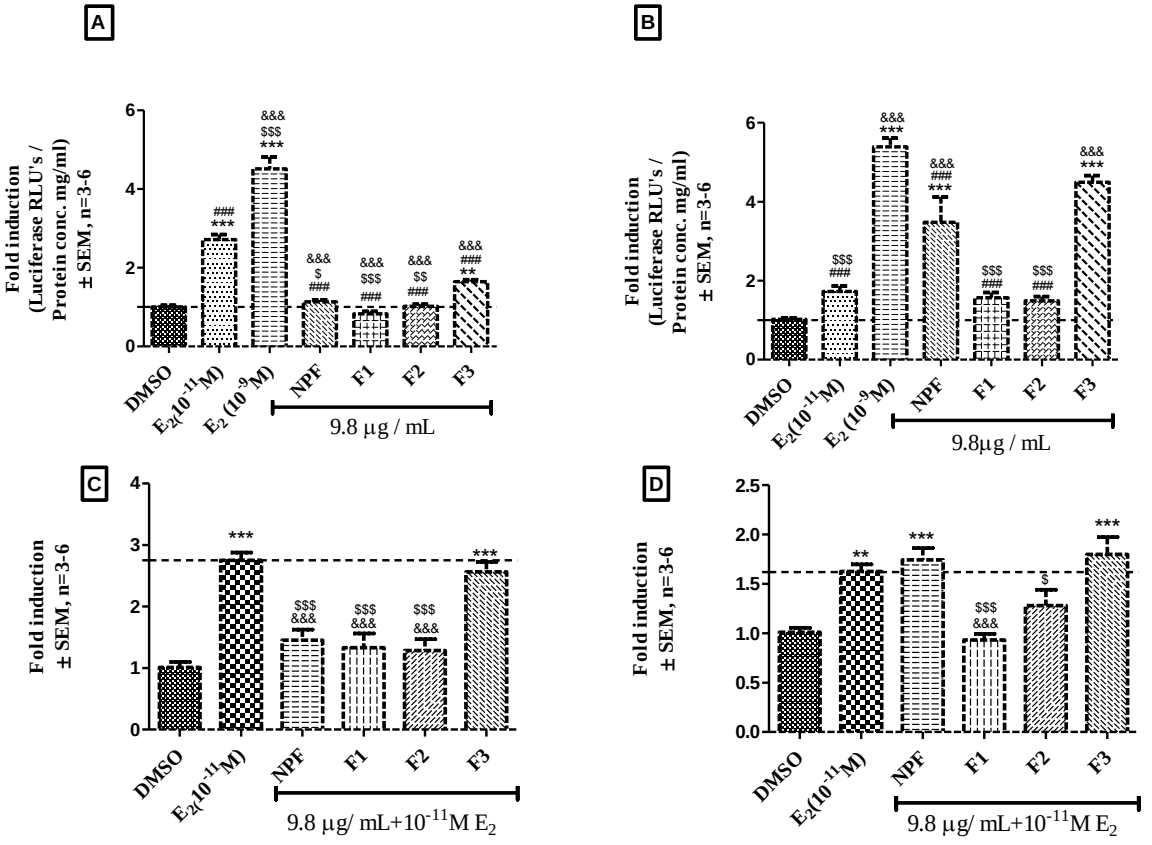

Figure 6. Transactivation studies investigating E_2, NPF from liquid-liquid fractionation and HPCCC fractions, F1-F3: A-B agonist mode; C-D antagonist mode (A) HEK293 cells transfected with pSG5-hERα, ERE.vit2.luc and pGL2-Basic were induced with 10^{-11}-10^{-9} M E_2, 9.8 µg/mL NPF, F1, F2 and F3 (B) HEK293 cells transfected with pSG5-hERβ, ERE.vit2.luc and pGL2-Basic were induced as in A. (C) HEK293 cells were transfected as in A induced with 10^{-11} M E_2, 9.8 µg/mL NPF, F1, F2 and F3, with the sample treatments co-administered with10^{-11}M E_2. (D) HEK293 cells were transfected as in B induced with 10^{-11} M E_2, 9.8 µg/mL NPF, F1, F2 and F3, with the fraction treatments co-administered with10^{-9} M E_2. For statistical analysis one-way ANOVA was used with Dunnett's Multiple Comparisons Test as post-test. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$. For all bars compared to 1 nM E_2 ### denotes $p < 0.001$, ## denotes $p < 0.01$ and # denotes $p < 0.05$. For all bars compared to F3 $$$ denotes $p < 0.001$, $$ denotes $p < 0.01$ and $ denotes $p < 0.05$. For all bars compared to 10 $^{-11}$M E_2 &&& denotes $p < 0.001$, && denotes $p < 0.01$ and & denotes $p < 0.05$. No symbol above bars indicates no significant differences compared to the above mentioned compared conditions.

Investigation of HPCCC fractions for ERβ agonism, an important estrogenic attribute, revealed that F3 acted as a potent ERβ agonist inducing a 4.5 fold induction (Fig. 6B). The induced activity was

significantly ($p < 0.001$) higher than that obtained with 10^{-11} M E_2, but not significantly different ($p > 0.05$) from 1 nM E_2 (Fig. 6B). Furthermore, it was also higher although not significantly so, than that obtained with NPF. In contrast, F1 and F2 showed no significant ERβ activation. In addition, none of the HPCCC fractions, with the exception of F1, displayed ERβ antagonism (Fig. 6D).

Evaluation of the effect HPCCC-obtained fractions on breast cancer cell proliferation revealed that similar to NPF, none of its fractions, with the exception of F3, induced significant breast cancer cell proliferation in the absence of E_2 (Fig. 7A). Furthermore, investigation of the final estrogenic attribute, antagonism of E_2-induced breast cancer cell proliferation, revealed that only F2, like NPF, displayed antagonism (Fig. 7B).

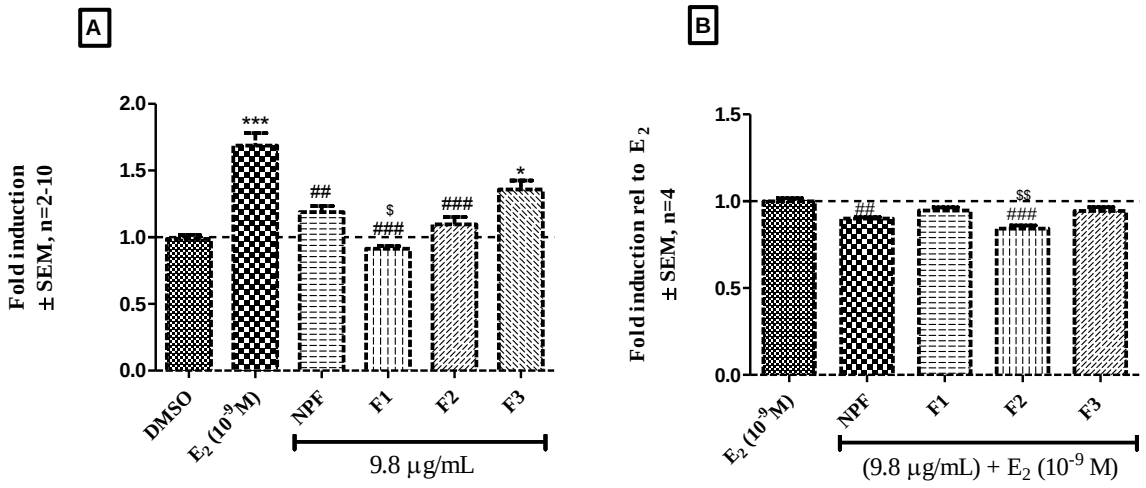

Figure 7. Proliferation studies investigating E_2, NPF and HPCCC fractions, F1-F3: A agonist mode; B antagonist mode MCF-7 BUS cells were serum starved for 24 h, where after cells were induced at 24 h, and re-induced at 48 h and 72 h. The assay was performed at 96 h. In (A) cells were induced with 10^{-9} M E_2, 9.8 µg/mL NPF, F1, F2 or F3. In (B) MCF-7 BUS cells were treated as in (A) with 10^{-9} M E_2, 9.8 µg/mL NPF, F1, F2 or F3, with the fraction treatments co-administered with10^{-9} M E_2. For statistical analysis one-way ANOVA was used with Dunnett's Multiple Comparisons Test as post-tests. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$. For all bars compared to 1 nM E_2 ### denotes $p < 0.001$, ## denotes $p < 0.01$ and # denotes $p < 0.05$. For all bars compared to F3 $$$ denotes $p < 0.001$, $$ denotes $p < 0.01$ and $ denotes $p < 0.05$. No symbol above bars indicates no significant differences compared to the above mentioned compared conditions.

LC-MS/MS analysis of the three HPCCC fractions (Table S3, Fig. S10D-F) revealed that each HPCCC fraction had a different polyphenolic profile, which was also different from NPF, due relative distribution of the major and minor polyphenolic compounds introduced by the HPCCC two-phase solvent system. Furthermore, in the quest for correspondance of major polyphenolic

compounds and compounds of interest with the positive estrogenic attributes of the fractions, we performed qHPLC analysis on these HPCCC fractions (Table 4, Fig S11D-F). F1 consisted mainly of dihydrochalcones (86.0% of major polyphenols in F1) and retained most (97%) of the dihydrochalcones recovered during HPCCC (Table 4). Of the dihydrochalcones in F1, phloretin-3',5'-di-*C*-glucoside was concentrated roughly 2-fold relative to NPF, while 3-hydroxyphloretin-3',5'-di-*C*-hexoside was present at the same concentration (Table 4). F1 also contained hesperidin, a minor flavanone component and eriocitrin, respectively, 0.19% and 13.3% of the quantified polyphenols in F1. In contrast to F1, 91% of all flavanones and 97% of iriflophenone-3-*C*-glucoside recovered during HPCCC eluted in F2. All the xanthones, flavones and phenolic acids eluted in F3 so that this fraction consisted of 62.9% xanthones, 31.8% flavones and 4.9% phenolic acids. Only trace amounts of the benzophenone, iriflophenone-3-*C*-glucoside, was present in F3.

Table 4: Individual polyphenol content as determined by qHPLC, concentration change relative to NPF, and % polyphenol present as per total quantified major polyphenols present in F1, F2 and F3.

	g/100 g test sample			Fold difference relative to NPF[a]			% polyphenol present[b]		
Polyphenol	**F1**	**F2**	**F3**	**F1**	**F2**	**F3**	**F1**	**F2**	**F3**
Mangiferin	Nd[c]	Nd	11.568	Nd	Nd	2.08	Nd	Nd	46.70
Isomangiferin	Nd	Nd	4.016	Nd	Nd	2.14	Nd	Nd	16.22
Scolymoside (luteolin-7-*O*-rutinoside)	Nd	Nd	7.669	Nd	Nd	2.07	Nd	Nd	30.97
Luteolin	Nd	Nd	0.205	Nd	Nd	1.95	Nd	Nd	0.83
Iriflophenone-3-*C*-glucoside	Nd	11.827	0.092	Nd	7.56	0.06	Nd	18.67	0.37
Phloretin-3', 5'-di-*C*-glucoside	6.075	0.691	Nd	1.76	0.20	Nd	67.31	1.09	Nd
3-Hydroxyphloretin-3',5'-di-*C*-hexoside	1.683	0.245	Nd	1.00	0.15	Nd	18.65	0.39	Nd
Eriocitin (eriodictyol-7-*O*-rutinoside)	1.185	11.148	Nd	0.56	5.24	Nd	13.13	17.60	Nd
Hesperidin (hesperetin-7-*O*-rutinoside)	0.082	39.446	Nd	0.02	7.36	Nd	0.19	62.26	Nd
Protocatechuic acid	Nd	Nd	0.647	Nd	Nd	1.86	Nd	Nd	2.6
***p*-Coumaric acid**	Nd	Nd	0.570	Nd	Nd	NA[d]	Nd	Nd	2.3

[a] Polyphenol concentration (g/100 g test sample) present in HPCCC fraction relative to polyphenol in NPF (Table 3), set as 1

[b] % of polyphenol present calculated relative to total concentration of major polyphenols in fraction

[c] Nd - not detected due to absence or very low amounts

[d] NA - value not available as *p*-coumaric acid co-elutes with 3-hydroxyphloretin-3',5'-di-*C*-hexoside where present

Thus in summary, analysis of the HPCCC fractions revealed separation of the previously demonstrated positive estrogenic attributes of NPF into separate fractions, with F1 and F2 acting as ERα antagonists, only F2 inducing antagonism of E_2 induced breast cancer cell proliferation and only F3 retaining ERβ agonist activity. Although the ERβ agonism displayed by F3 was robust and significantly higher than that of 10^{-11} M E_2, it also displayed weak ERα agonism. Also interestingly, for the first time in this study, ERβ antagonism was observed, as induced by F1. F1 was the dihydrochalcone-rich fraction, and also contained ca. 31% of the eriocitrin in the HPCCC fractions. This compound has been shown to have estrogenic activity [61]. F2 was the flavanone and benzophenone-rich fraction, of which only the benzophenone, iriflophenone-3-*C*-glucoside, and eriocitrin may possibly display phytoestrogenic activity [61,75,76]. Amongst the F3 polyphenolic constituents, only luteolin, present in low quantities, is a known phytoestrogen [81]. Scolymoside, a luteolin rutinoside, may be phytoestrogenic, although it has not previously been tested. If not phytoestrogenic, it could potentially have activity *in vivo* when the sugar moiety is removed to form luteolin [82].

3.3.1.4 Purification schedule of SM6Met B1-4Mix fractionation

Having discussed each of the fractionation steps separately, we now proceed to a discussion of the overall purification schedule of the fractionation outcomes. As results for both estrogenic activity and polyphenol content are available, we constructed purification tables of polyphenols present in fractions (Table 5) and estrogenic activity of fractions (Table 6). For the construction of the estrogenic activity purification table it would have been preferable to have quantitative data on efficacy and potency, however, as we did not construct dose response curves, we have to rely on the individual values obtained in our assays. Despite this drawback we assayed all fractions at the same concentration (9.8 µg/mL) and thus the results may be considered as a type of specific activity value.

Table 5 Purification table of polyphenolic compounds (constructed from results presented in Figure 1 and Tables 2-4)

	SM6Met B1-4Mix				PF				NPF			
Polyphenol	**g/100 g SM6Met**	**Fractionation Yield (%)[a]**	**SA (g/100 g test sample)[b]**	**PuF[c]**	**g/100 g SM6Met[d]**	**Fractionat-ion Yield (%)**	**SA (g/100 test sample)**	**PuF**	**g/100 g SM6Met**	**Fractionation Yield (%)**	**SA (g/100 g test sample)**	**PuF**
Mangiferin	1.899	100.0	1.899	1.00	0.063	3.3	0.094	0.05	1.564	82.3	5.559	2.93
Isomangiferin	0.645	100.0	0.645	1.00	0.033	5.1	0.048	0.08	0.528	81.9	1.879	2.91
Scolymoside	1.289	100.0	1.289	1.00	0.073	5.7	0.108	0.08	1.044	81.0	3.710	2.88
Luteolin	0.040	100.0	0.04	1.00	0.000	0.0	Nd[f]	nd	0.030	74.2	0.105	2.63
Iriflophenone-3-*C*-glucoside	0.669	100.0	0.669	1.00	0.155	23.2	0.230	0.34	0.440	65.9	1.565	2.34
3-Hydroxyphloretin-3',5'-di-*C*-hexoside	0.700	100.0	0.7	1.00	0.154	21.9	0.227	0.32	0.474	67.7	1.686	2.41
Phloretin-3',5'-di-*C*-glucoside-	1.278	100.0	1.278	1.00	0.170	13.3	0.251	0.20	0.972	76.0	3.456	2.70
Eriocitrin (eriodictyol-7-*O*-rutinoside)	0.846	100.0	0.846	1.00	0.127	15.0	0.187	0.22	0.599	70.8	2.129	2.52
Hesperidin (hesperitin-7-*O*-rutinoside)	2.049	100.0	2.049	1.00	0.248	12.1	0.367	0.18	1.507	73.5	5.356	2.61
***p*-Coumaric acid**	co-elution	co-elution[e]	co-elution	1.00	0.000	0.0	nd	nd	co-elution	co-elution	co-elution	co-elution
Protocatechuic acid	0.113	100.0	0.113	1.00	0.000	0.0	nd	nd	0.098	86.2	0.347	3.07
	F1				F2				F3			
Polyphenol	**g/100 g SM6Met**	**Fractionation Yield (%)**	**SA(g/100 g test sample)**	**PuF**	**g/100 g SM6Met**	**Fractionat-ion Yield (%)**	**SA(g/100 g test sample)**	**PuF**	**g/100 g SM6Met**	**Fractionat-ion Yield (%)**	**SA(g/100 g test sample)**	**PuF**
Mangiferin	0.000	0.0	Nd	Nd	0.000	0.0	nd	nd	1.063	55.9	11.565	6.09
Isomangiferin	0.000	0.0	Nd	Nd	0.000	0.0	nd	nd	0.369	57.2	4.016	6.23
Scolymoside (luteolin-7-*O*-rutinoside)	0.000	0.0	Nd	Nd	0.000	0.0	nd	nd	0.705	54.7	7.669	5.95
Luteolin	0.000	0.0	Nd	Nd	0.000	0.0	nd	nd	0.019	47.4	0.205	5.14
Iriflophenone-3-*C*-glucoside	0.000	0.0	Nd	Nd	0.318	47.5	11.827	17.68	0.008	1.3	0.092	0.14
3-Hydroxyphloretin-3',5'-di-*C*-hexoside	0.189	27.0	1.683	2.40	0.007	0.9	0.245	0.35	0.000	0.0	nd	nd
Phloretin-3',5'-di-*C*-glucoside-	0.684	53.5	6.075	4.75	0.019	1.5	0.691	0.54	0.000	0.0	nd	nd
Eriocitrin (eriodictyol-7-*O*-rutinoside)	0.133	15.8	1.185	1.40	0.299	35.4	11.148	13.18	0.000	0.0	nd	nd
Hesperidin (hesperitin-7-*O*-rutinoside)	0.009	0.5	0.082	0.04	1.060	51.7	39.446	19.25	0.000	0.0	nd	nd
***p*-Coumaric acid**	0.000	0.0	Nd	Nd	0.000	0.0	nd	nd	0.052	NA[g]	0.570	NA
Protocatechuic acid	0.000	0.0	Nd	Nd	0.000	0.0	nd	nd	0.059	52.4	0.647	5.722

[a] Fractionation yield was calculated relative to initial concentration of polyphenol in SM6Met B1-4Mix, which was set to 100%; for example for mangiferin in PF (0.063/1.899) x 100 = 3.3%

[b] SA refers to specific activity, which is the concentration of polyphenol per 100 g test sample
[c] PuF refers to purification factor (SA of compound in fraction/ SA of compound in SM6Met B1-4Mix)
[d] g polyphenol in fraction if 100 g SM6Met B1-4Mix was used for fractionation (Fig. 1) for example mangiferin in NPF: (5.559/100) x 28.14 g (as in Fig. 1)=1.564 g
[e]Co-elution refers to the fact that the polyphenol co-elutes with 3-hydroxyphloretin-3',5'-di-*C*-hexoside
[f] Nd - polyphenols were not detected due to absence or very low amounts
[g] NA - not available as *p*-coumaric acid co-elutes with 3-hydroxyphloretin-3',5'-di-*C*-hexoside where present

Table 6: Purification table of estrogenic activity

	ERα				ERβ			MTT		
	Agonist activity		Antagonist activity		Agonist activity		Antagonist activity	Agonist activity	Antagonist activity	
	Fold[a]	PuF[b]	% decrease[c]	PuF	Fold	PuF	% decrease	Fold	% decrease	PuF
B1-4Mix	1.8 ###	1.0	33% ###	1.0	2.2 ###	1.0	—	—	18% ##	1.0
PF	—		26% #	0.8	—	—	—	—	17% ##	0.94
NPF	—		30% ##	0.9	2.3 ##	1.1	—	—	17% ##	0.94
F1	—		52% ###	1.6	—	—	43% ###	—	—	—
F2	—		53% ###	1.6	—	—	—	—	16% ###	0.90
F3	1.7 ###	0.9	—		4.5 ns	2.1	—	1.4 ns	—	—

[a] Fold-induction relative to solvent (Figs. 2- 7)
[b] PuF refers to purification factor (SA of compound in fraction/ SA of compound in SM6Met B1-4Mix)
[c] Percentage decrease in induction relative to 10^{-11} M E_2 set as 100% for promoter reporter studies and 10^{-9} M E_2 set as 100% for proliferation studies (Figs. 2-7)
# indicates statistical difference compared to E_2, where ns displays activity not statistically different from E_2
— refers to no activity: for agonist mode this implies no statistical difference from solvent, while for antagonist mode it implies no statistical difference from 10^{-11} M $E_2$for promoter reporter studies and 10^{-9} M E_2 for proliferation studies.

The initial liquid-liquid fractionation of SM6Met B1-4Mix produced two fractions, PF and NPF, which was produced with an overall (g/g) yield of 96%, split into 68% for PF and 28% for NPF. Fractionation resulted in an increase in the purification of major polyphenols in NPF as reflected by purification factor (PuF) values ranging from 2.34 – 3.07 (Table 5). In terms of estrogenic activity, all the positive estrogenic attributes of the initial SM6Met B1-4Mix were retained in NPF (Table 6), whereas PF retained only ERα antagonism and antagonism of E_2-induced breast cancer cell proliferation. Despite the concentration of major polyphenolic compounds in NPF we did not observe a significant increase in estrogenic activity as compared to that observed for SM6Met B1-4Mix.

Subsequent HPCCC fractionation of NPF produced three fractions, F1, F2 and F3, comprising an overall yield of 82%, split into 40% for fraction F1, 9.6% for fraction F2, and 32.7% for fraction F3. Considering fractionation of the polyphenols, the dihydrochalcones predominantly eluted in F1, while the benzophenone and flavanones eluted in F2, and the xanthones, flavones and polyphenolic acids eluted in F3 (Table 5). Specifically, phloretin-3',5'-di-*C*-glucoside and 3-hydroxyphloretin-3',5'-di-*C*-hexoside were concentrated 4.8 fold and 2.4 fold, respectively, in fraction F1, while iriflophenone-3-*C*-glucoside and the flavanones, eriocitrin and hesperidin, were concentrated 17.7, 13.2, and 19.3 fold, respectively in fraction F2. The xanthones, mangiferin and isomangiferin, were concentrated 6.0 and 6.2 fold in fraction F3, while the flavones, scolymoside and luteolin, were concentrated 6.0 and 5.1 fold, respectively. Although, the phenolic acids, *p*-coumaric acid and protocatechuic acid, displayed similar specific activity values in fraction F3, the purification factor could only be determined for protocatechuic acid (5.7 fold), as the concentration of *p*-coumaric could not be determined in the initial SM6Met B1-4Mix, due to co-elution with 3-hydroxyphloretin-3',5'-di-*C*-hexoside. In terms of estrogenic activity, HPCCC resulted in the divergence, and in some cases amplification, of the positive estrogenic attributes of the initial SM6Met B1-4Mix and NPF. Specifically, ERα antagonism was amplified (1.6 fold) in fractions F1 and F2, whereas it was absent from fraction F3, while ERβ agonism was only observed in fraction

F3, where it was amplified 2 fold. The third positive estrogenic attribute, antagonism of E_2-induced breast cancer cell proliferation, was only retained in fraction F2, the only fraction that displayed pure ERα antagonism, an attribute previously shown to be anti-proliferative in breast cancer cells [44]. Although, fraction F1 also displayed ERα antagonism, the fact that, in addition, it also displayed ERβ antagonism, possibly explains the loss of antagonism of E_2-induced breast cancer cell proliferation when the roles of the ER-subtypes in breast cancer cell proliferation are considered [44].

In summary, the liquid-liquid fractionation concentrated all known major quantified polyphenolic compounds thereby retaining the positive estrogenic attributes in NPF, while the subsequent HPCCC of NPF separated not only the major classes of quantified polyphenolic compounds, but also the desired positive estrogenic attributes into three fractions.

F3 was chosen for further evaluation as ERβ agonism is considered important in the development of a phytoestrogenic nutraceutical for women in menopause due to the known fact that ERβ agonism is anti-proliferative in breast cancer [44], while recent studies have indicated that ERβ agonists may alleviate several menopausal symptoms [83,84].

3.3.2 Evaluation of the estrogenic activity of individual polyphenols in F3 and reconstituted F3

Having quantified the major polyphenolic compounds in HPCCC fraction F3 (Table 4), we wanted to investigate whether reconstitution of the HPCCC fraction F3 using only the major phenolic compounds quantified would display the same activity as the isolated HPCCC fraction. We reconstituted the F3 fraction using the concentrations of major phenolic compounds that would be present in 9.8 µg/mL of extract (Table 7).

The HPCCC fraction F3 previously showed potent ERβ agonist activity (Fig. 6B), which was also displayed upon re-investigation (Fig. 8B), with the HPCCC fraction F3 inducing a 3.5 fold induction of an ERE-containing promoter reporter via ERβ.

Table 7: Individual polyphenol content as per 9.8 µg HPCCC fraction F3

Polyphenol	HPCCC fraction F3 polyphenol content	
	µg/9.8 µg extract	% polyphenol present[b]
Mangiferin	1.113	46.7
Isomangiferin	0.394	16.2
Luteolin	0.020	0.8
Scolymoside (luteolin-7-*O*-rutinoside)	0.752	31.0
Iriflophenone-3-*C*-glucoside	0.009	0.4
Phloretin-3', 5'-di-*C*-glucoside	Nd[a]	Nd
3-Hydroxyphloretin-3',5'-di-*C*-hexoside	Nd	Nd
Eriocitin	Nd	Nd
Hesperidin (hesperitin-7-*O*-rutinoside)	Nd	Nd
Protocatechuic acid	0.063	2.6
***p*-Coumaric acid**	0.056	2.3

[a] Nd - polyphenol not detected
[b] % of polyphenol present calculated relative to total concentration of major polyphenols in extract

The reconstituted F3 major compound mixture (F3M) induced a 2.6 fold induction via ERβ, which was unfortunately significantly ($p < 0.01$) lower than HPCCC fraction F3. Despite this, the ERβ antagonism experiment (Fig. 8D) confirmed that both HPCCC fraction F3 and F3M are ERβ agonists displaying, in the presence of 10^{-11} M E_2, an agonism significantly ($p < 0.05$) higher than 10^{-11} M E_2. Previously, HPCCC fraction F3 displayed weak ERα agonism (Fig. 6A), which was also displayed upon re-investigation (Fig. 8A), with HPCCC fraction F3 inducing a 1.8 fold induction of an ERE-containing promoter reporter via ERα. F3M, however, induced a significantly ($p < 0.001$) higher induction (2.7 fold) via ERα. The ERα agonism of HPCCC fraction F3, but not F3M, was significantly lower than 10^{-11} M E_2.

Together these results indicate that neither the desired ERβ agonist activity, nor the weak ERα agonism, present in the HPCCC fraction F3, could be fully recapitulated by reconstituting only the major polyphenolic compounds present in HPCCC fraction F3. We should, however, keep in mind that neither scolymoside nor iriflophenone 3-*C*-glucoside used to reconstitute F3M were pure compounds, but rather enriched HPCCC fractions, thus, the concentration of these compounds in F3M were underestimated, which may have contributed to the discrepancy between the HPCCC fraction F3 and F3M results. Specifically, the HPCCC enriched fraction of scolymoside contained only 20.5 g (Fig. S3) scolymoside per 100 g extract, while the HPCCC enriched iriflophenone-3-*C*-glucoside fraction contained only 32 g iriflophenone-3-*C*-glucoside per 100 g extract (Fig. S4).

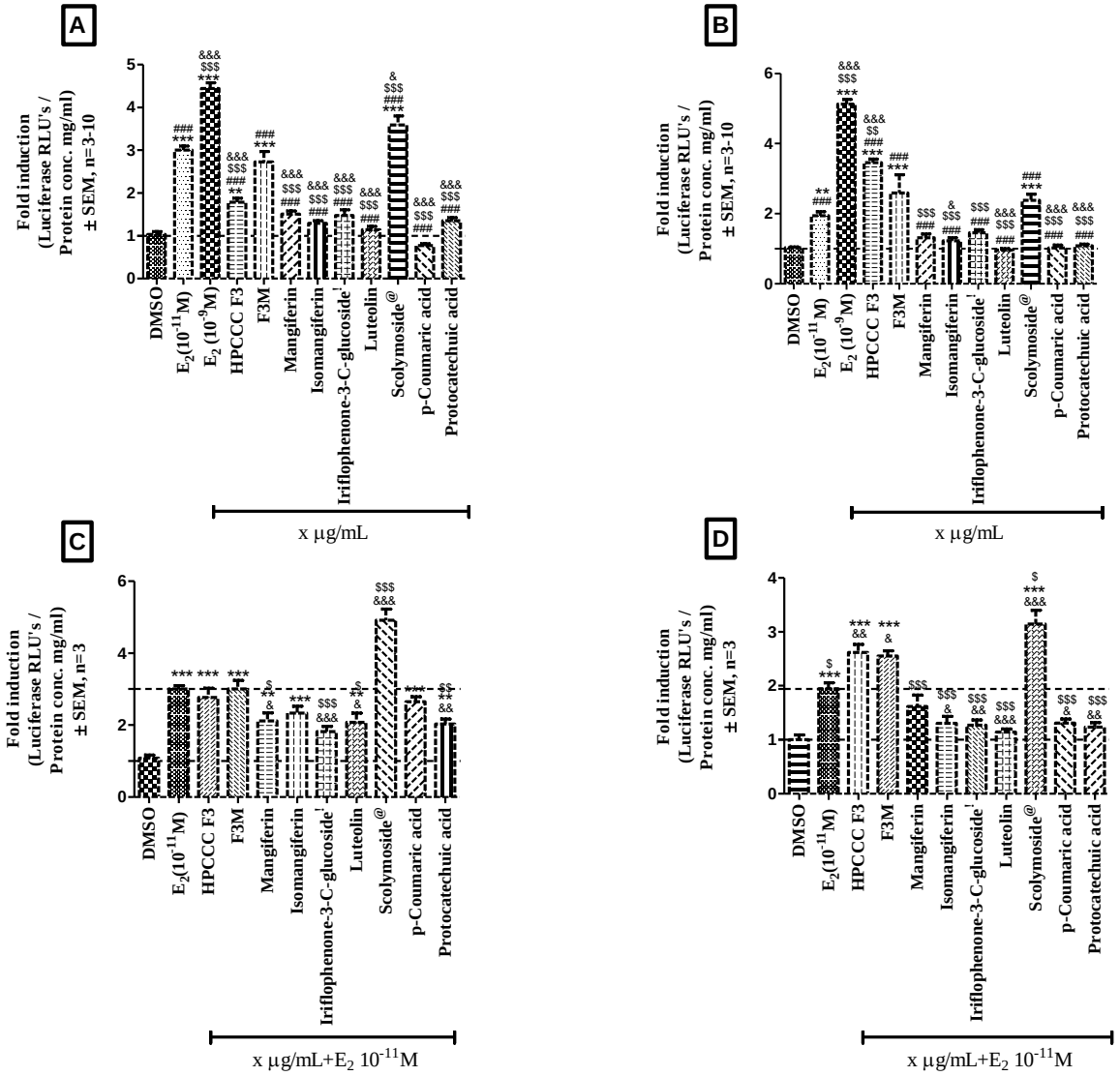

Figure 8. Transactivation studies investigating E_2, HPCCC fraction F3, reconstituted fraction F3 (F3M) and compounds as in HPCCC fraction F3: A-B Agonist Mode; C-D Antagonist Mode. (A) HEK293 cells transfected with pSG5-hERα, ERE.vit2.luc and pGL2-Basic cells were induced with 10^{-11}-10^{-9} M E_2, 9.8 µg/mL F3 (HPCCC F3 and F3M); and 1.133 µg mangiferin, 0.394 µg isomangiferin, 0.056 µg *p*-coumaric acid, 0.063 µg protocatechuic acid, 0.752 µg scolymoside enriched HPCCC fraction (Fig. S3), 0.0201 µg luteolin or 0.0090 µg iriflophenone-3-*C*-glucoside enriched HPCCC fraction (Fig. S4), as in 9.8 µg F3 (Table 7). (B) HEK293 cells transfected with pSG5-hERβ, ERE.vit2.luc and pGL2-Basic were induced as in A. (C) HEK293 cells were transfected and induced as in A with HPCCC F3, F3M, HPCCC-rich fractions and compounds, administered in the presence of 10^{-11} M E_2. (D) HEK293 cells were transfected as in B induced with HPCCC F3, F3M, HPCCC-rich fractions and compounds, administered in the presence of 10^{-11} M E_2. For statistical analysis one-way ANOVA was used with Dunnett's Multiple Comparisons Test as post-test. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$. For all bars compared to 1nM E_2 ### denotes $p < 0.001$, ## denotes $p < 0.01$ and # denotes $p < 0.05$. For all bars compared to F3M $$$ denotes $p < 0.001$, $$ denotes $p < 0.01$ and $ denotes $p < 0.05$. For all bars compared to 10^{-11}M E_2 &&& denotes $p < 0.001$, && denotes $p < 0.01$ and & denotes $p < 0.05$. No symbol above bars indicates no significant differences compared to control. @ refers to the luteolin-7-*O*-rutinoside enriched fraction. ! refers to the iriflophenone-3-*C*-glucoside enriched fraction.

Alternatively, other minor compounds present in HPCCC fraction F3 may be involved in modulating towards greater ERβ agonism and lesser ERα agonism as evaluated via the ERE-

containing promoter reporter assay. Despite the differences observed between the HPCCC fraction F3 and F3M in the subtype specific promoter reporter assays, no significant difference was observed in the breast cancer cell proliferation assay, with both fractions displaying weak and significantly lower ($p < 0.001$) induction of cell proliferation than 1nM E_2 (Fig. 9A).

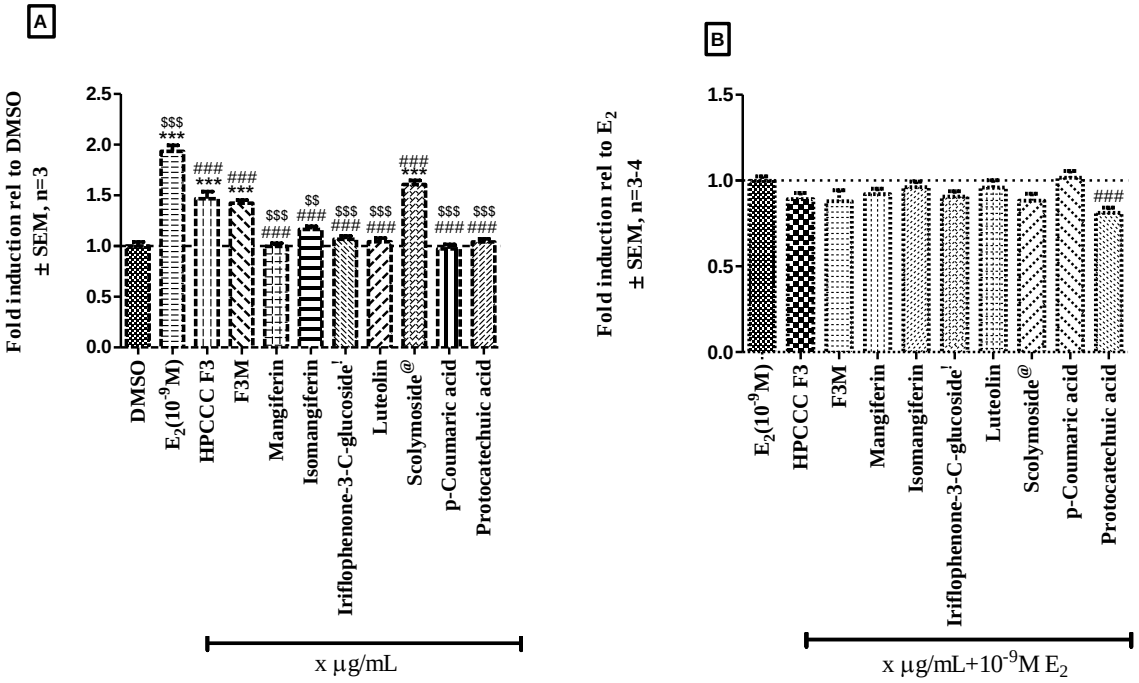

Figure 9. Proliferation studies investigating investigating E_2, HPCCC fraction F3, reconstituted fraction F3 (F3M) and compounds as in HPCCC F3 (F3M): A agonist mode; B antagonist mode. MCF-7 BUS cells were serum starved for 24 h, where after cells were induced at 24 h, and re-induced at 48 h and 72 h, where after the assay was performed at 96 h. In (A) cells were induced with 10^{-9} M E_2, 9.8 µg/mL F3 (HPCCC F3 and F3M) and 1.133 µg mangiferin, 0.394 µg isomangiferin, 0.056 µg *p*-coumaric acid, 0.063 µg protocatechuic acid, 0.752 µg scolymoside enriched HPCCC fraction, 0.0201 µg luteolin and 0.0090 µg iriflophenone-3-*C*-glucoside HPCCC enriched fraction, as in 9.8 µg HPCCC F3 (Table 7). In (B) MCF-7 BUS cells were treated as in (A) with the fractions and compounds, administered in the presence of 10^{-9} M E_2. For statistical analysis one-way ANOVA was used with Dunnett's Multiple Comparison Test as post-test. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$. For all bars compared to 1 nM E_2 ### denotes $p < 0.001$, ## denotes $p < 0.01$ and # denotes $p < 0.05$. For all bars compared to F3M $$$ denotes $p < 0.001$, $$ denotes $p < 0.01$ and $ denotes $p < 0.05$. No symbol above bars indicates no significant differences compared to control. @ refers to the luteolin-7-*O*-rutinoside enriched fraction. ! refers to the iriflophenone-3-*C*-glucoside enriched fraction.

Despite the differences between HPCCC fraction F3 and F3M, it is interesting to note the contribution of the individual major polyphenolic compounds to ER-subtype specific activity (Fig. 8) compared to F3M. The desired ERβ agonism appears to be mostly explained by the presence of the scolymoside enriched fraction (Fig. 8B), which displayed similar ($p > 0.05$) ERβ agonism as F3M. Interestingly enough, mangiferin, isomangiferin, luteolin, *p*-coumaric acid, and

protocatechuic acid, at the concentrations of the pure compounds present in F3M, as well as the iriflophenone-3-*C*-glucoside HPCCC enriched fraction, displayed ERβ antagonism (Fig. 8D), which is not reflected as ERβ antagonism of F3M, nor in reduced ERβ agonism of F3M relative to scolymoside (Fig. 8B). Scolymoside or other compounds present in the scolymoside enriched fraction, is not only an ERβ agonist, but also displayed ERα agonism (Fig. 8A), which in contrast to that found via ERβ, is significantly higher ($p < 0.01$) than observed for F3M. Here we find that ERα antagonism (Fig. 8C) of other major compounds (mangiferin, iriflophenone-3-*C*-glucoside (or other minor compounds in the enriched fraction), luteolin, and protocatechuic acid), at the concentrations present in F3M, appeared to be responsible for tempering the strong ERα agonism of scolymoside to result in the lower ERα agonism seen for F3M (Fig. 8A). Weak induction of breast cancer cell proliferation was observed for the scolymoside enriched fraction (Fig. 9A), which although not significantly different from F3M, is slightly higher. The downward modulation of the proliferative effect of the scolymoside enriched HPCCC fraction observed in the final F3M mixture could be ascribed to the significant ($p < 0.001$) antagonism of protocatechuic acid (Fig. 9B).

In summary, reconstitution of the major polyphenolic compounds of the HPCCC fraction F3 in F3M did not result in similar activities in the ER-subtype specific ERE-containing promoter reporter assays. The discrepancy in the results (Figs. 8A&B) suggested that minor compounds in the HPCCC fraction F3 would have to act as ERα antagonists and ERβ agonists. Amongst the individual major compounds, scolymoside or minor compounds in the scolymoside enriched fraction, appears to be the most likely candidates to elicit the observed ER agonism of F3M. Interestingly, scolymoside, a rutinoside of luteolin, has not previously been investigated for ER-subtype specific activity, and thus this study is the first to show that it may behave as both an ERα and ERβ agonist. However, we should keep in mind that pure scolymoside was not tested, but rather an enriched HPCCC fraction, and thus any conclusions drawn concerning this fraction should await validation with pure scolymoside.

3.4 Discussion

Menopausal women have shown great interest in commercial phytoestrogenic nutraceuticals, as it is seen as a safer alternative to traditional HRT [85-91]. *Cyclopia* shows potential for the development of a phytoestrogenic nutraceutical [61,62]. More recently SM6Met, a sequential methanol extract from a *Cyclopia subternata* harvesting, M6, showed the highest potency of tested *Cyclopia* extracts with regards to phytoestrogenic properties and displayed positive estrogenic attributes, such as ERα antagonism, ERβ agonsim and antagonism of E_2-induced breast cancer cell proliferation [63,64]. In the current study, these positive estrogenic attributes were used in activity-guided fractionation of SM6Met with the hope of identifying marker compound(s) responsible for these attributes to be used for quality control, screening, and in future plant breeding programs.

Activity-guided fractionation is commonly used to identify compounds within mixtures responsible for specific activity profiles [92-94]. In the field of estrogenic nutraceuticals, activity-guided fractionation was most successfully employed to isolate the ERβ-subtype specific agonist, liquiritigenin, from MF101, a plant extract containing 22 herbs, which has ERβ selective properties [94]. In the case of SM6Met the desired positive estrogenic attributes are more complex in that both ERβ agonism, as well as ERα antagonism was targeted. The existence of a compound displaying these desired estrogenic attributes seemed possible as the R,R enantiomer of 5, 11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (R,R-THC) has been shown to be both an ERα agonist and an ERβ antagonist [42,43,95]. Our results, however, suggest that it is unlikely that the ERβ agonism as well as the ERα antagonism of SM6Met may be attributed to a single compound as we showed that ERα antagonism was only observed for HPCCC fractions F1 and F2, but not F3, while ERβ agonism was only observed for HPCCC fraction F3. The difference in activity was as a result of the differential fractionation of the major polyphenolic compounds in NPF into the individual HPCCC fractions. Specifically, the HPCCC fraction F1 consisted mainly of dihydrochalcones, the HPCCC fraction F2 of mainly flavanones and the benzophenone, and the HPCCC fraction F3 of mainly xanthones, flavones, and phenolic acids.

The two dihydrochalcones, phloretin-3',5'-di-*C*-glucoside and 3-hydroxyphloretin-3',5'-di-*C*-hexoside, present in HPCCC fraction F1 have not been tested for estrogenic activity, although a previous study did correlate estrogenic activity with the presence of phloretin-3',5'-di-*C*-glucoside in SM6Met, which was previously unidentified, and designated as unknown 2 [63]. Although present at lower levels than the dihydrochalcones, one of the other major polyphenolic compounds found in HPCCC fraction F1, eriocitrin, has been shown to bind both ERα and ERβ [61], however, no information concerning agonism or antagonism is available. Thus, although no definitive conclusions regarding the specific polyphenolic compounds responsible for the observed ERα and ERβ antagonism of HPCCC fraction F1 may be drawn, the fact that eriocitrin was also present in HPCCC fraction F2, which also displayed ERα antagonism, is intriguing. Evidence supporting the possible ERα antagonism of eriocitrin is that it binds to ERα [61], and is present in HPCCC fractions F1 and F2, but not in HPCCC fraction F3. However, the fact that there is no quantitative difference in the level of ERα antagonism between HPCCC fraction F1 and F2, despite F2 containing ten times more eriocitrin than F1, argues against eriocitrin being solely responsible for the observed ERα antagonism. No other compounds that could possibly explain the ERα antagonism and antagonism of E_2-induced breast cancer cell proliferation of HPCCC fraction F2 appeared to be present, as hesperidin, the most abundant polyphenol present in F2, does not bind ERα or ERβ while iriflophenone, the aglycone of iriflophenone-3-*C*-glucoside, the second most abundant polyphenol in F2, has previously been shown to be proliferative in breast cancer cells [75,76], a finding we could not reproduce with the iriflophenone-3-*C*-glucoside enriched fraction (Fig. 6A). Our own results with the iriflophenone-3-*C*-glucoside enriched fraction, however, suggested that iriflophenone-3-*C*-glucoside, or the suite of minor compounds accompanying iriflophenone-3-*C*-glucoside in the enriched fraction (Fig. S4), could act as ERα and ERβ antagonists (Figs 8C&D). Studies have indicated that iriflophenone-3-*C*-β-glucoside may exert synergistic effects with other compounds in SM6Met such as mangiferin and hesperidin [96,97] which has been linked to anti-diabetic and cholesterol-lowering effects seen in rat models [98]. It

could be interesting to investigate synergistic estrogenic effects of iriflophenone-3-*C*-glucoside and hesperidin as both are present in the iriflophenone-3-*C*-glucoside enriched fraction (Fig. S4) and in F2. Our own results (Fig. 8) implicated the scolymoside enriched HPCCC fraction as the primary candidate responsible for the robust ERβ agonism of HPCCC fraction F3. Scolymoside has not previously been tested for estrogenicity. Our study showed that the scolymoside-enriched HPCCC fraction displayed not only ERβ agonism, but also ERα agonism, which is likely due to the presence of scolymoside, but, may also be due to the presence of the other minor compounds in the scolymoside enriched HPCCC fraction (Fig. S3). Luteolin, the other flavone and an aglycone of scolymoside, present in HPCCC fraction F3, has been much more extensively studied. Although luteolin is a known phytoestrogen [81] showing preferential binding to ERβ over ERα, transactivating an ERE-containing promoter reporter via both ER-subtypes, though more potently via ERα, and antagonising E_2-induced breast cancer cell proliferation [62,77,81], our results suggested that at the concentration present in HPCCC fraction F3, luteolin displayed none of the above mentioned estrogenic properties. We did, however, observe ERα and ERβ antagonism, which we postulated could be responsible for tempering the ERα agonism of the scolymoside enriched HPCCC fraction towards the lowered agonism observed for F3M. The most abundant class of polyphenols in HPCCC fraction F3 were the xanthones, mangiferin and isomangiferin. Mangiferin does not bind to the ER-subtypes [59], nor does it transactivate an ERE-containing promoter reporter via either ER-subtype [62], which corresponds with our findings (Figs. 8A&B). Antagonism via the ER-subtypes has not previously been investigated; our results indicated that mangiferin acts as an ERα, but not ERβ antagonist (Fig. 8C). Furthermore, although mangiferin has been shown to have anti-tumour properties against MCF-7 breast cancer cells by regulating matrix metalloproteinases, the epithelial to mesenchymal transition, and the β-catenin signalling pathway [99], our results indicted no antagonism of E_2-induced breast cancer cell proliferation at the concentration of mangiferin present in HPCCC fraction F3 (Fig. 9B). Our studies indicated that the other xanthone, isomangiferin, a regio-isomer of mangiferin, acted as an ERβ antagonist at the

concentration present in HPCCC fraction F3. To our knowledge no other studies investigating the phytoestrogenic properties of isomangiferin have been done. The phenolic acids, *p*-coumaric acid and protocatechuic acid, present in lower amounts in HPCCC fraction F3, are unlikely to contribute to ERβ agonism displayed by this fraction, as in our study they did not transactivate via either of the ER-subtypes (Figs. 8A&B), and previous studies have shown that *p*-coumaric acid was not estrogenic in a rat uterotrophic model [80]. Interestingly, both phenolic acids displayed ERβ antagonism, while in addition, the protocatechuic acid displayed ERα antagonism (Fig 8C) and antagonism of E_2-induced breast cancer cell proliferation (Fig. 9B).

It should be noted that all discussions regarding scolymoside and iriflophenone-3-*C*-glucoside should, however, be viewed in the light of the fact that these compounds were not tested as pure compounds, but rather as enriched fractions. The scolymoside enriched fraction also contained trace amounts of mangiferin and isomangiferin and low amounts of hesperidin , while the iriflophenone-3-*C*-glucoside enriched fraction also contained trace amounts of mangiferin, isomangiferin, eriocitrin and narirutin, and low amounts of maclurin-3-*C*-glucoside and hesperidin (Figs. S3&4). It is thus possible that the effects observed for these enriched fractions are actually due to the combination of compounds in the enriched fractions.

Future work should focus on fine-tuning the polyphenol composition of F3M to bring activity more in line with HPCCC fraction F3. This may be accomplished by, for example, including minor compounds identified in HPCCC fraction F3 (Table S3). Fine-tuning of F3M may allow for identification of the suite of compounds that together display robust and specific ERβ agonism, without ERα agonism. In addition, reconstitution of HPCCC fractions F1 and F2 should also be considered to identify the marker compounds or suite of compounds responsible for ERα and ERβ antagonism. Furthermore, consideration should be given to expanding the array of estrogenic assays, to include *in vitro* assays such as, whole cell binding, determination of ERα and ERβ levels, ERα and ERβ hetero- and homo-dimerization, and microarray analyses to evaluate effects on

endogenous genes and *in vivo* assays such as an immature rat uterine model or a rat breast cancer model. Furthermore, estrogenic activity assays to be conducted in the future should also include investigation of test samples in the presence and absence of the ER antagonist, ICI 182780, in order to determine whether the response induced by a test sample is indeed via the ER only. In addition, future research should include dose response curves for all extracts and fractions as this will allow one to acquire the relevant efficacy and potency data, which will allow for a more accurate activity analysis.

In conclusion, the fact that the positive estrogenic attributes of interest, ERα antagonism, ERβ agonism, and antagonsim of E_2-induced breast cancer cell proliferation, separated into different fractions following HPCCC fractionation of NPF, indicates that correlating one compound with all the positive estrogenic attributes is highly unlikely. This supports the idea that several of the polyphenols present in SM6Met or the NPF may have synergistic, additive or antagonistic effects in conferring these desired estrogenic traits. The concept of formulations consisting of intelligent mixtures of natural products to treat diseases has become a popular thrust of recent studies [100-105], and thus, future studies will include focussing on the isolation or production of a mixture of compounds, an "intelligent" mixture, where compounds show additive, synergistic or antagonistic effects to design a nutraceutical product to user demand of estrogenic activity.

3.5 Bibliography

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3.6 Supplementary Data

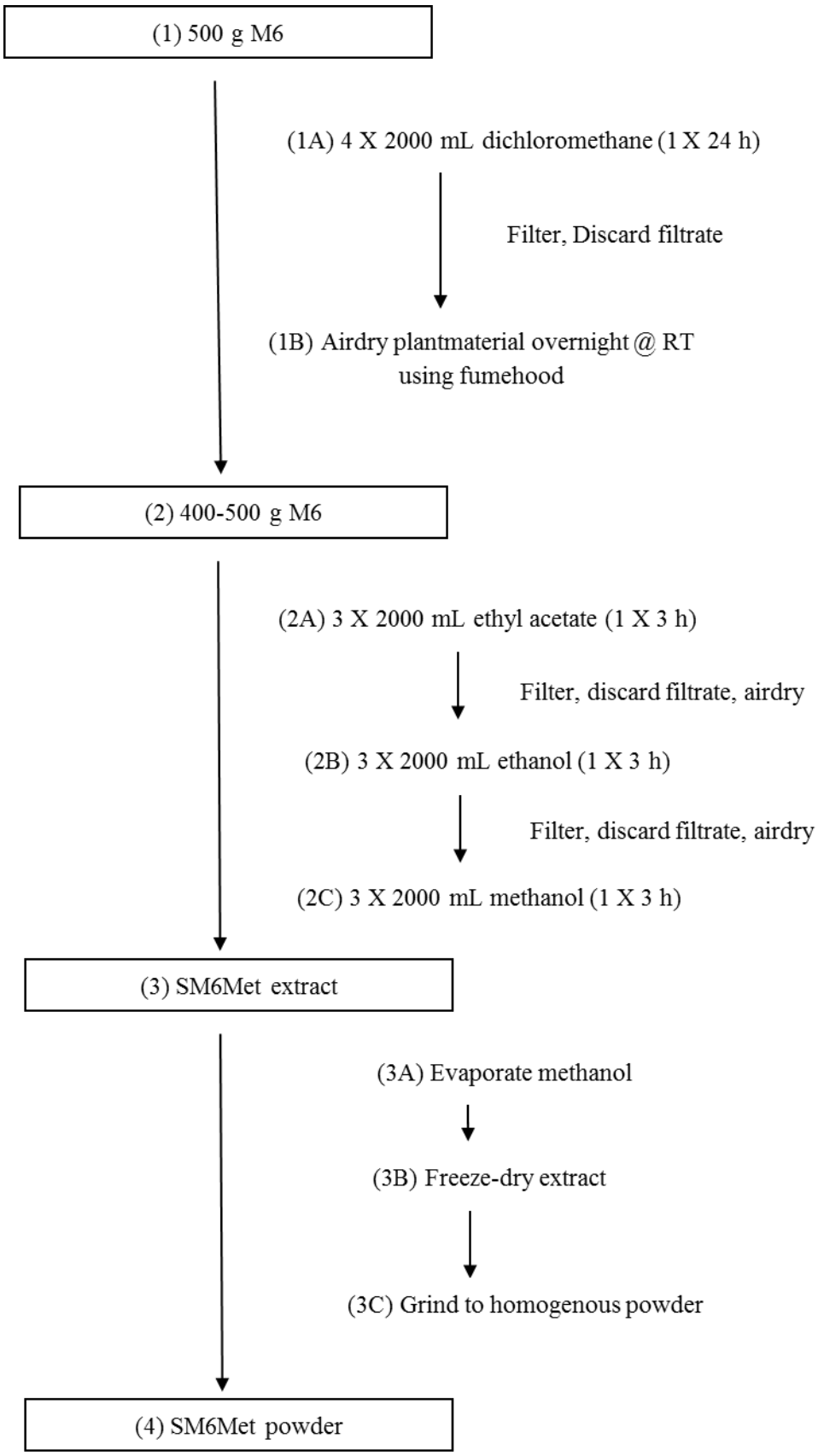


Figure S1. Production of SM6Met. SM6Met was produced as adapted from Mfenyana *et al.* [63].

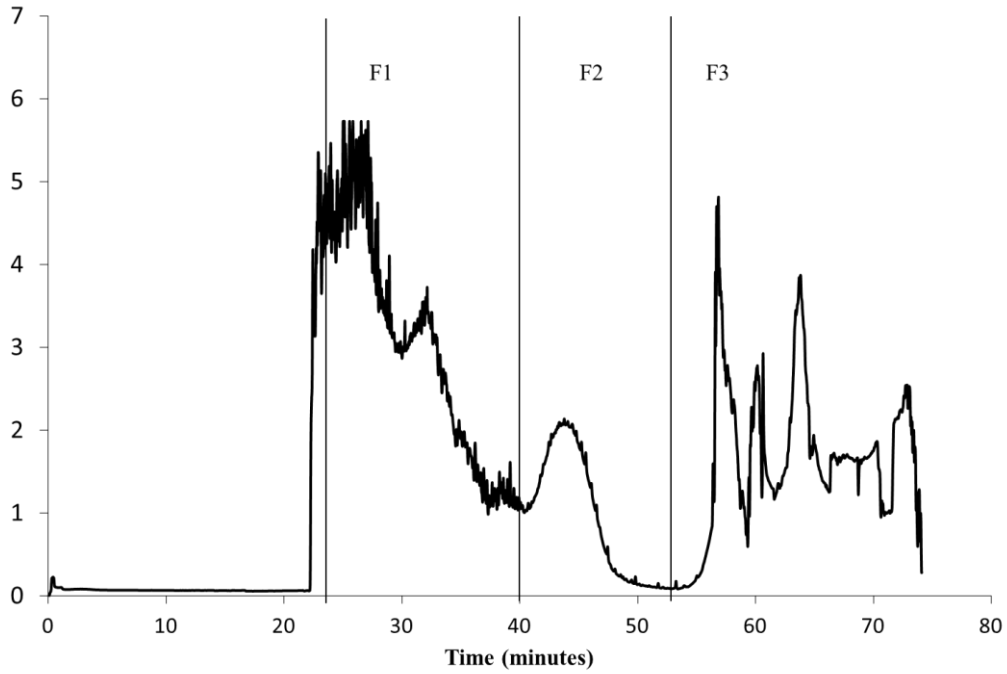


Figure S2. Representative high performance counter-current chromatogram (HPCCC) illustrating the fractionation of the non-polar fraction (NPF), whereby specific fractions were pooled to form F1 (24-40 min), F2 (41-52 min) and F3 (52-75 min).

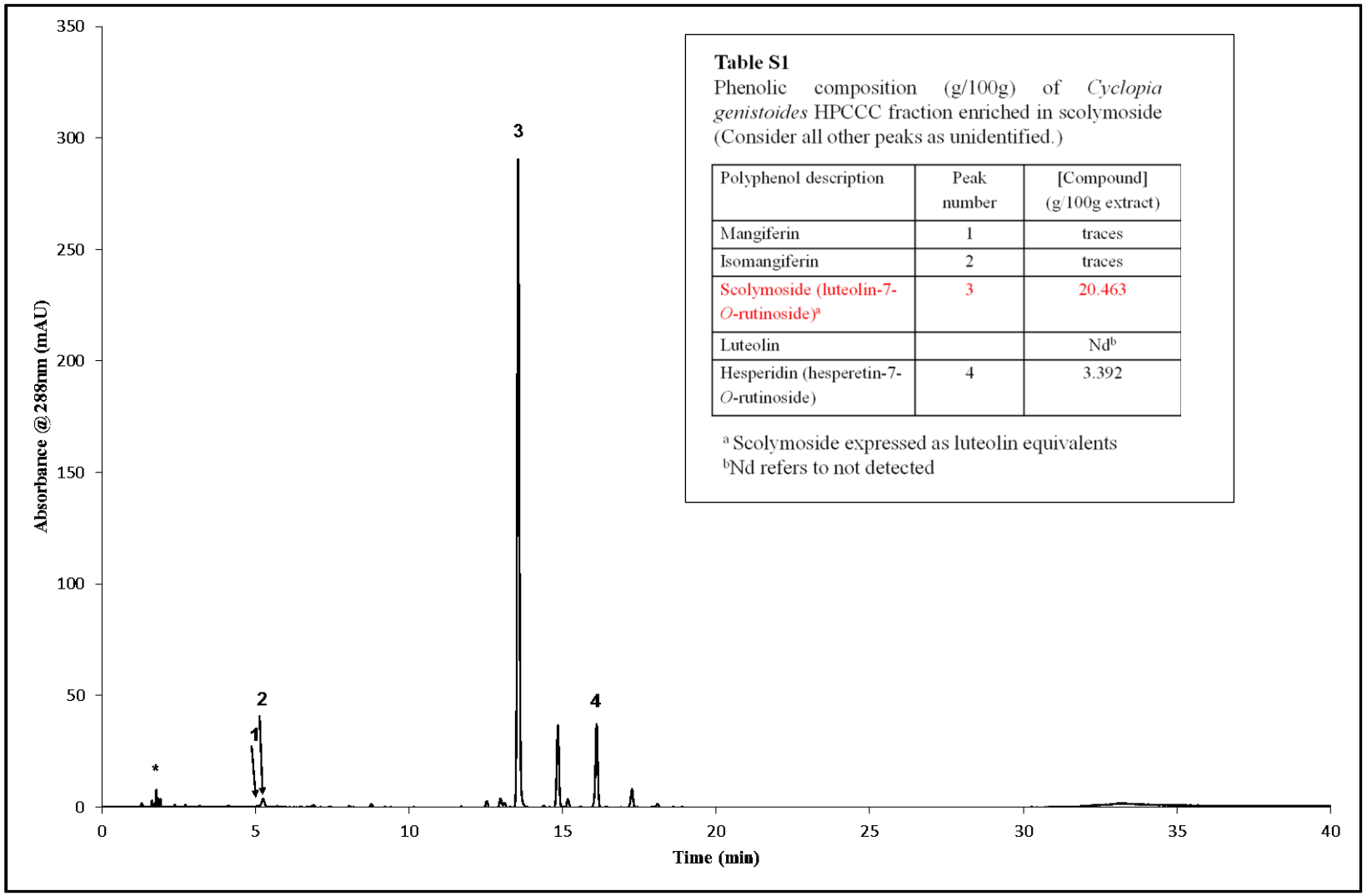


Table S1
Phenolic composition (g/100g) of *Cyclopia genistoides* HPCCC fraction enriched in scolymoside (Consider all other peaks as unidentified.)

Polyphenol description	Peak number	[Compound] (g/100g extract)
Mangiferin	1	traces
Isomangiferin	2	traces
Scolymoside (luteolin-7-*O*-rutinoside)[a]	3	20.463
Luteolin		Nd[b]
Hesperidin (hesperetin-7-*O*-rutinoside)	4	3.392

[a] Scolymoside expressed as luteolin equivalents
[b]Nd refers to not detected

Figure S3. HPLC-DAD chromatogram of a high performance counter-current chromatography (HPCCC) fraction enriched in scolymoside. * indicates ascorbic acid added to prevent oxidation during analysis. Numbers correspond with Table S1. Consider all other peaks as unidentified.

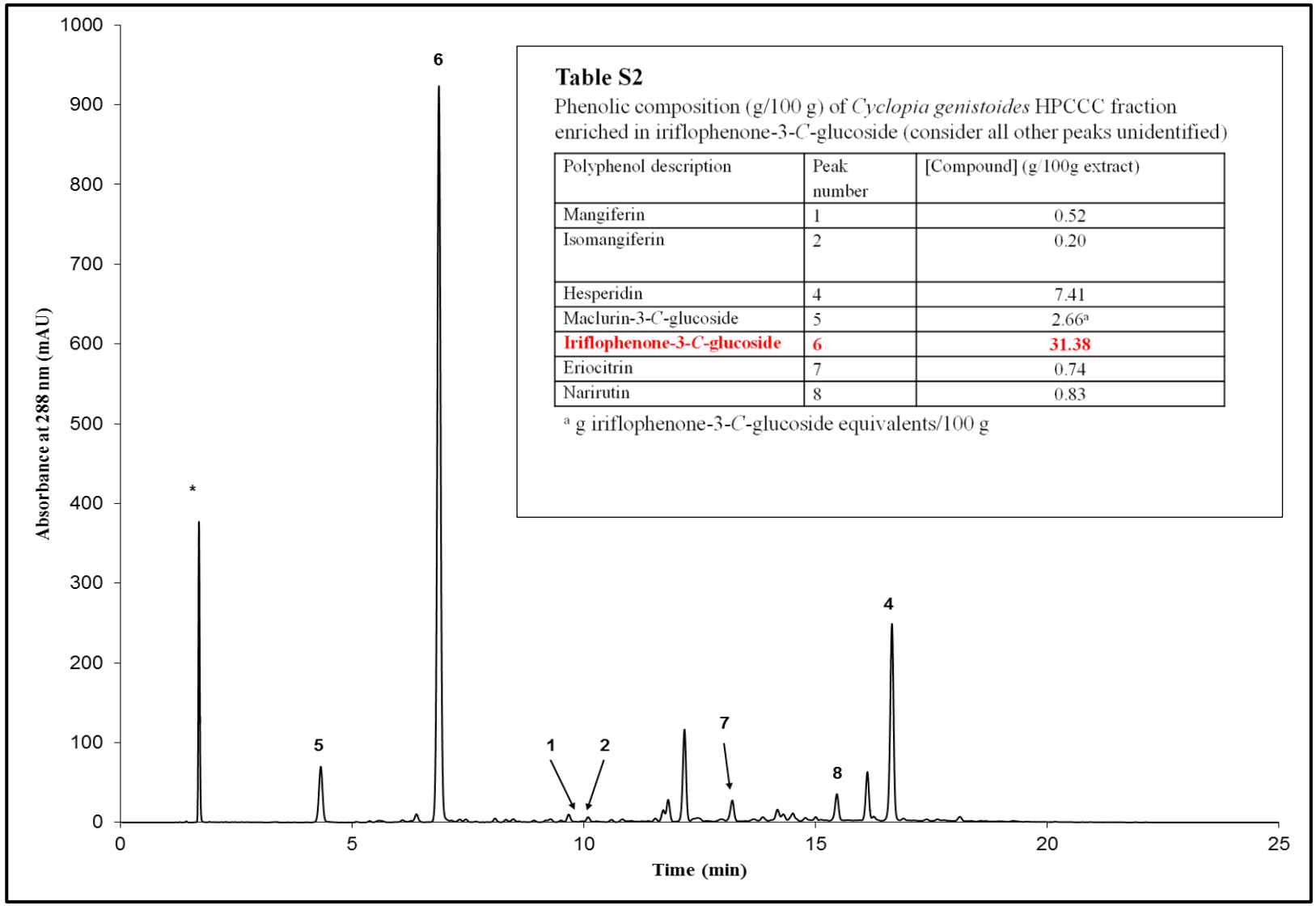


Table S2

Phenolic composition (g/100 g) of *Cyclopia genistoides* HPCCC fraction enriched in iriflophenone-3-*C*-glucoside (consider all other peaks unidentified)

Polyphenol description	Peak number	[Compound] (g/100g extract)
Mangiferin	1	0.52
Isomangiferin	2	0.20
Hesperidin	4	7.41
Maclurin-3-*C*-glucoside	5	2.66[a]
Iriflophenone-3-*C*-glucoside	**6**	**31.38**
Eriocitrin	7	0.74
Narirutin	8	0.83

[a] g iriflophenone-3-*C*-glucoside equivalents/100 g

Figure S4. HPLC-DAD chromatogram of high performance counter-current chromatography (HPCCC) fraction enriched in iriflophenone-3-*C*-glucoside. * indicates ascorbic acid added to prevent oxidation during analysis. Numbers correspond with Table S2. Consider all other peaks as unidentified.

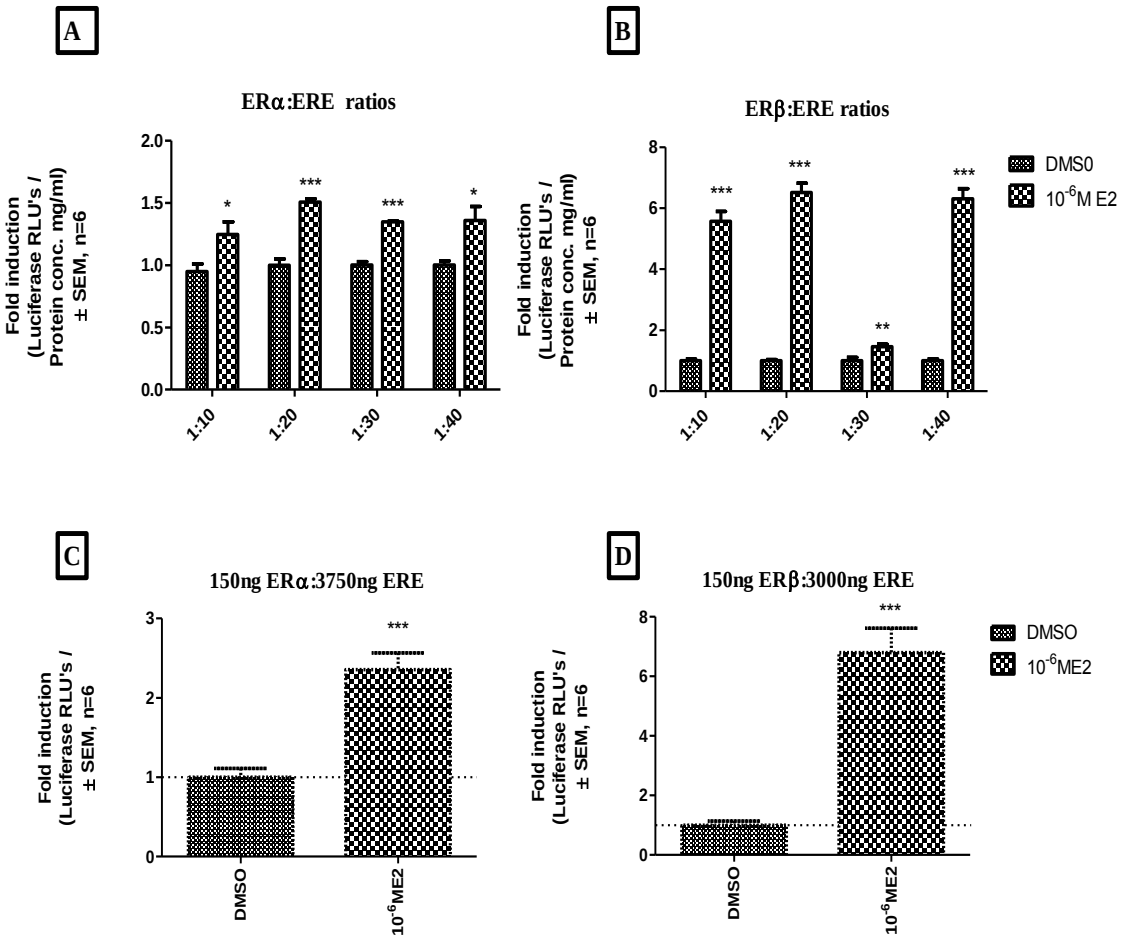


Figure S5. Optimisation of HEK 293 transfection. HEK 293 cells were transfected with varying ratios of 150 ng pSG5-hERα or 150 ng pSG5-hERβ to ERE.vit2.luc (1:10, 1:20, 1:30 and 1:40) with the addition of pGL-2-Basic in each instance to reach a total DNA amount of 6150 ng. In A) ERα:ERE DNA ratios, whereas in B) ERβ:ERE ratios were tested using normal sterile 10 cm and 24-well tissue culture plates. In C) and subsequently for all following transfections a ratio of 1:25 ERα:ERE was chosen coupled with the usage of sterile cell-bind 10 cm and 24-well tissue culture plates, whereas in D) and subsequently for all following transfections a ratio of 1:20 ERβ:ERE was chosen coupled with the usage of sterile cell-bind 10 cm and 24-well tissue culture plates. For statistical analysis one-way ANOVA was used with an unpaired t-test. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$

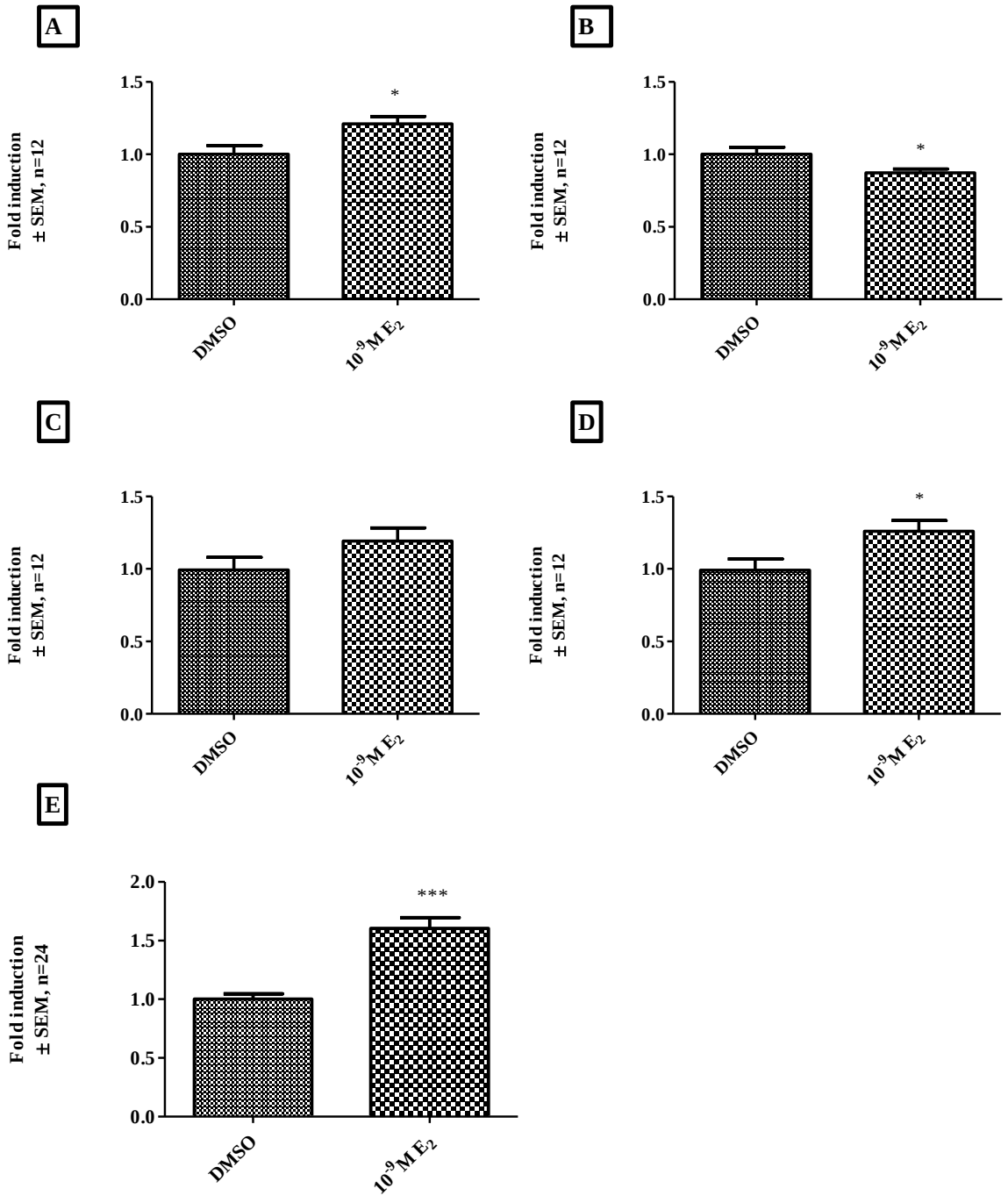


Figure S6. Optimisation of the treatment schedule for optimal E_2-induced breast cancer cell proliferation responsiveness. A) Induction of breast cancer cell (MCF-7 BUS)proliferation 48 h after the first treatment with E_2, B) 72 h after treatment, C) 48 h after the first treatment, with re-treatment at 24 h, D) 72 h after the first treatment with re-treatment at 24 h, E) 72 h with re-treatment at 24 h and 48 h. The treatment schedule for all following proliferation studies was chosen as 72 h with re-treatment at 24 h and 48 h as it produced the most optimal responsiveness of E_2-induced breast cancer cell proliferation. Proliferation was measured by means of the MTT assay. For statistical analysis one-way ANOVA was used with an unpaired t-test. For all bars compared to DMSO *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$

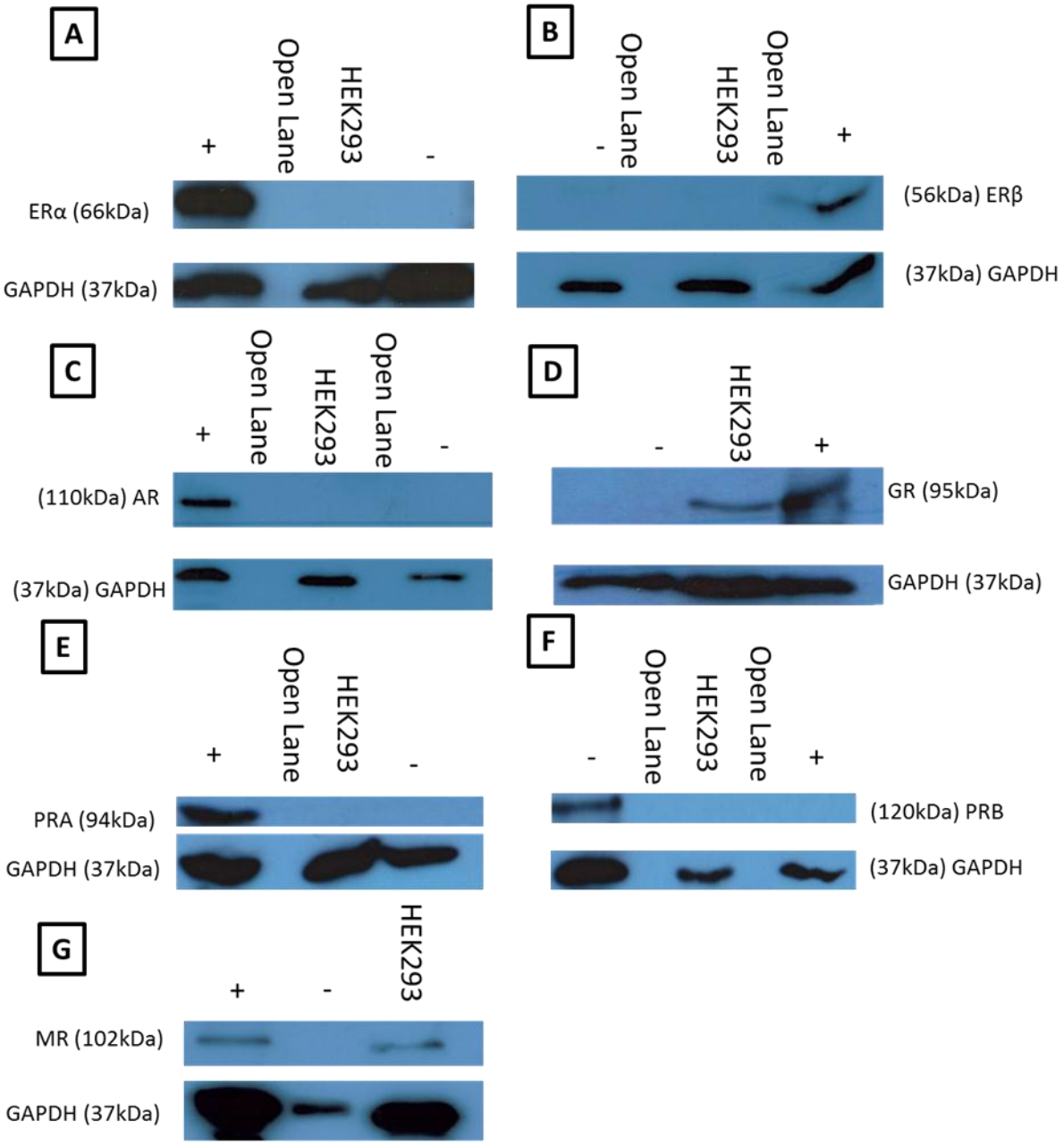


Figure S7. Steroid receptor characterisation of the HEK293 cell line. Western Blot analysis reveals the absence of ERα (A), ERβ (B), AR (C), PRA (E), PRB (F) and the presence of hGRα (D) and MR (G) in the HEK293 cell line. + indicates positive control for the steroid receptor which is the lysate obtained from COS-1 cells transfected with the cognate receptor, - indicates the negative control for the steroid receptor consisting of lysate from untransfected COS-1 cells, except for MR where it refers to an Ect1/E6E7 cell lysate. HEK293 refers to the HEK293 lysate used for HEK293 steroid receptor characterisation.

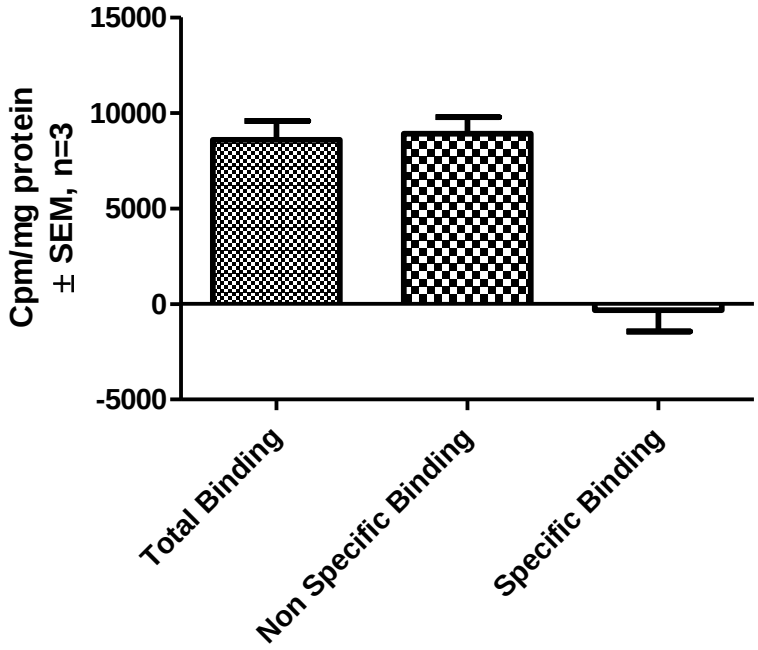


Figure S8. Estrogen receptor characterisation of HEK293 cells using a whole cell binding assay. For estrogen receptor characterisation HEK293 cells were incubated with 20 nM radio-labelled E_2 (2,4,6,7-[^{3}H]17-β-oestradiol, specific activity 100 Ci/mmol), and DMSO, which served as the total binding condition. In addition, the cells were incubated with 20 nM radio-labelled E_2 and 10^{-5} M unlabelled E_2, which served as the non-specific binding condition. Specific binding was calculated by subtracting the non-specific binding from the total binding. The graph shows results of three independent experiments. For statistical analysis, Student's t-test was used, which indicated no significant differences between total binding and non-specific binding.

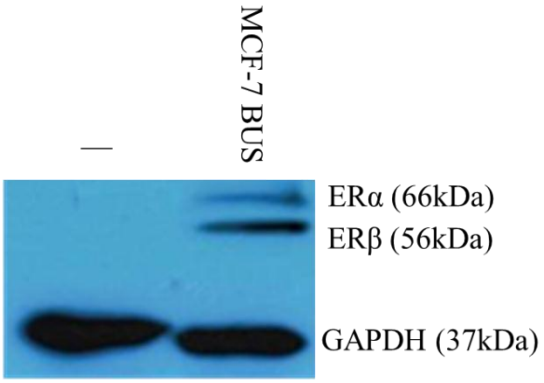
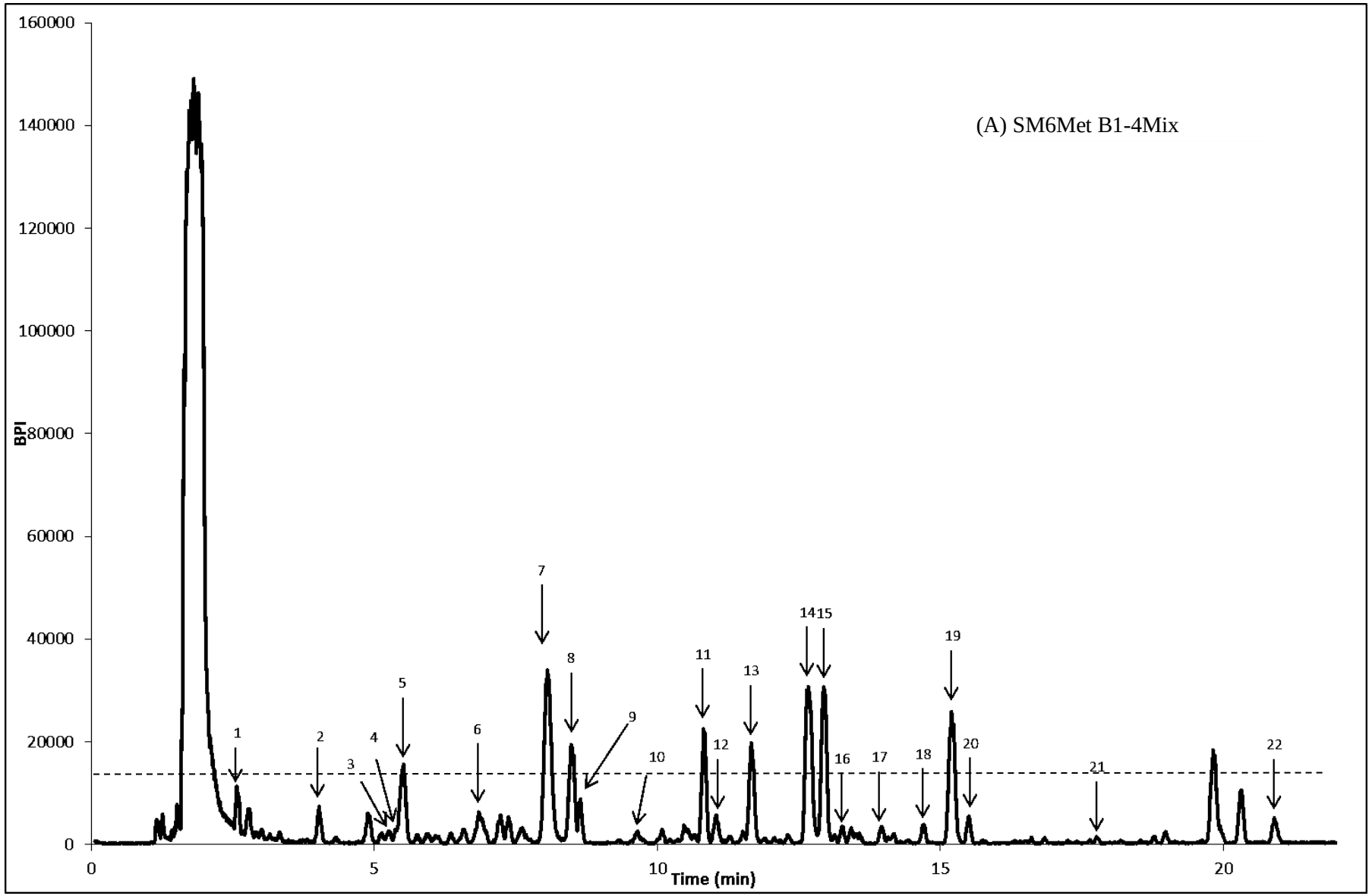
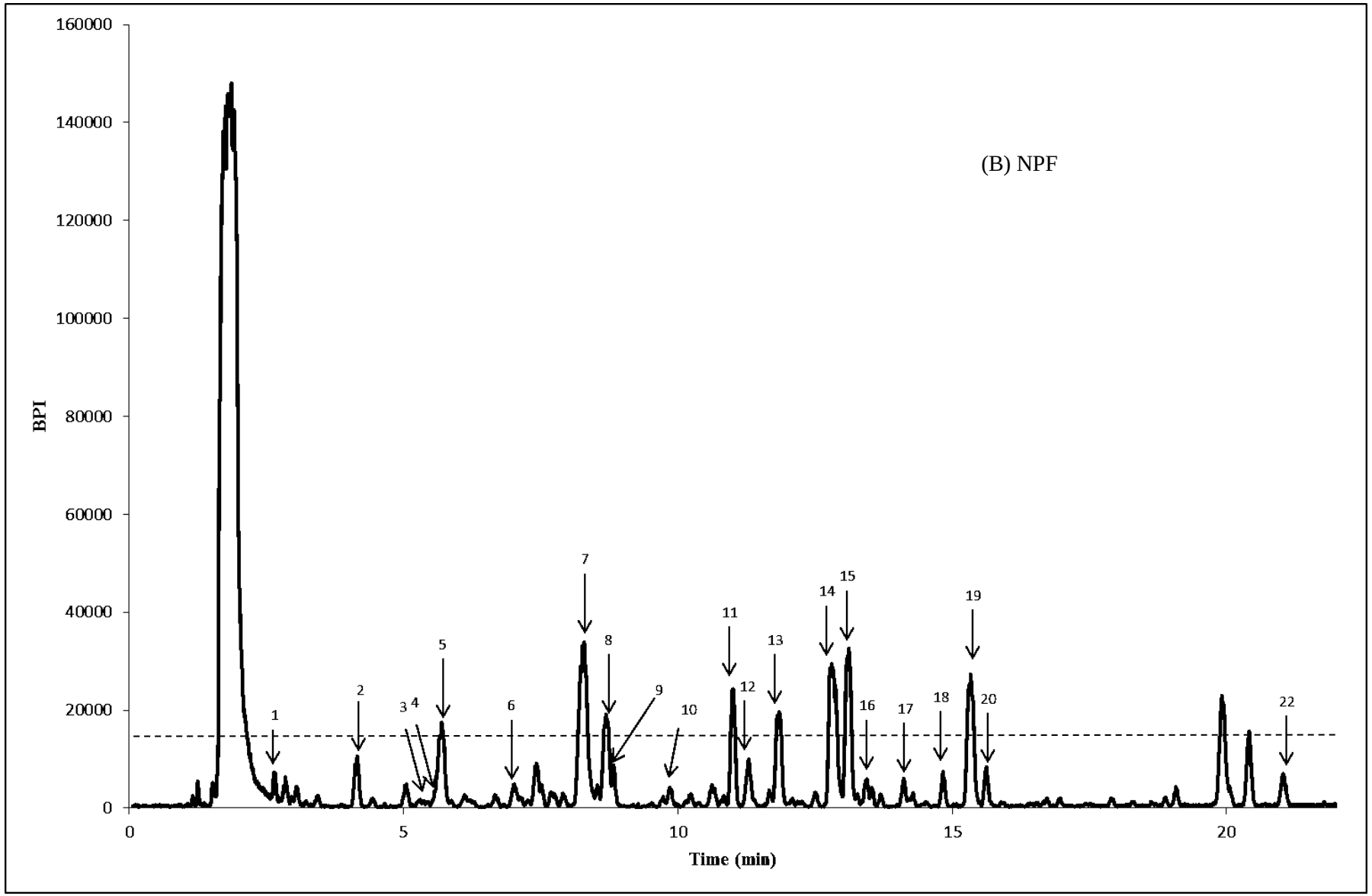
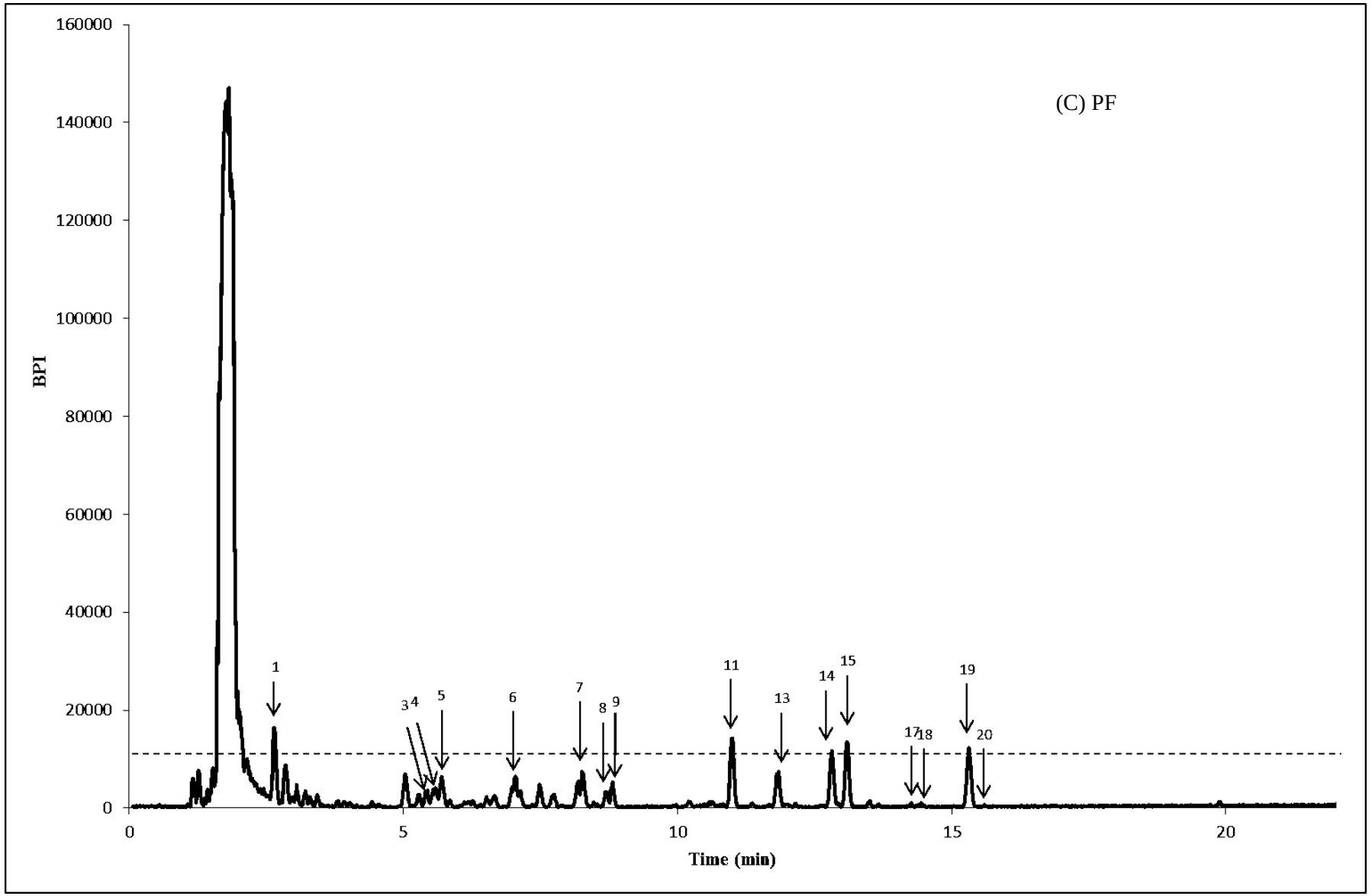
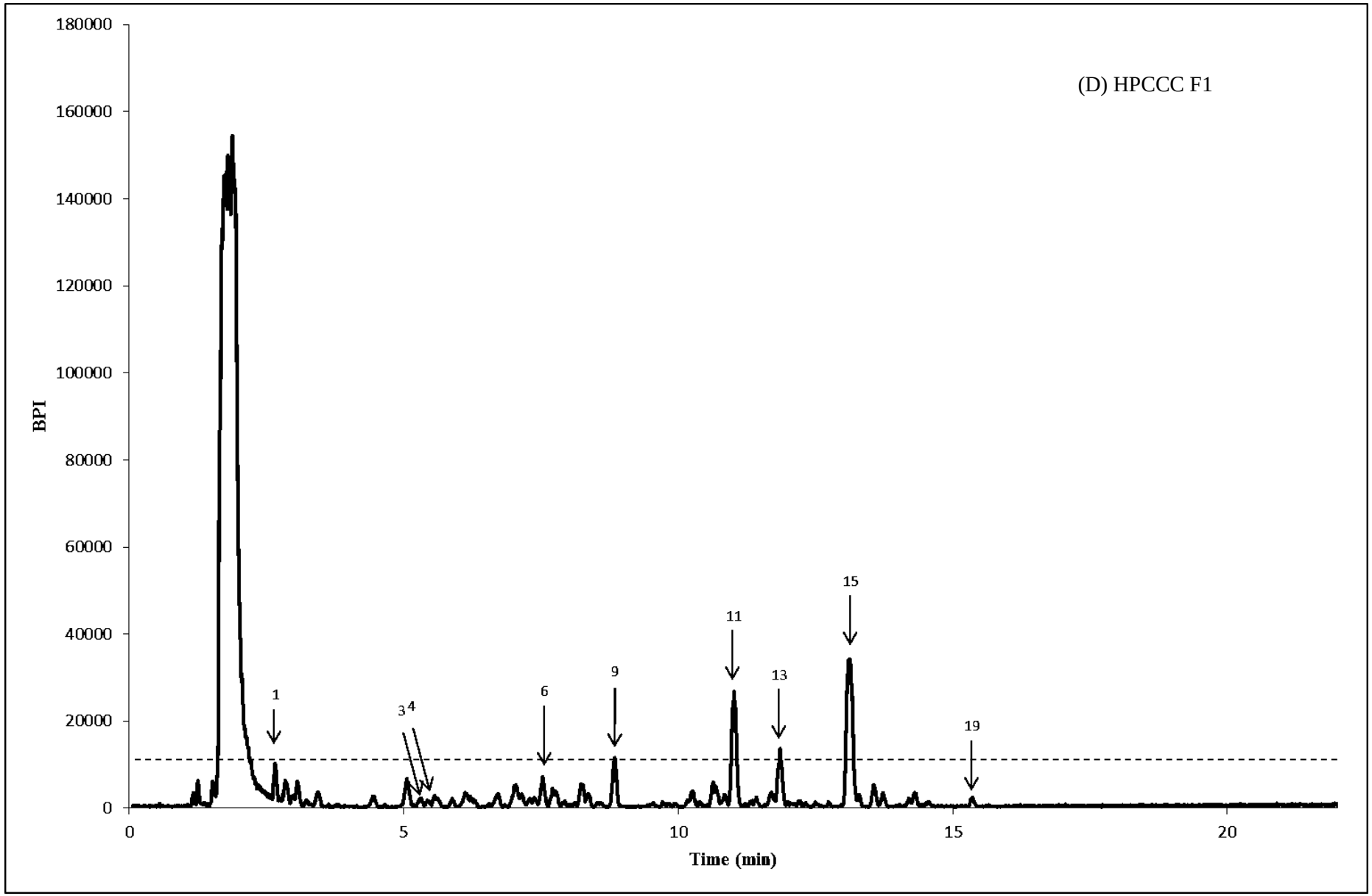


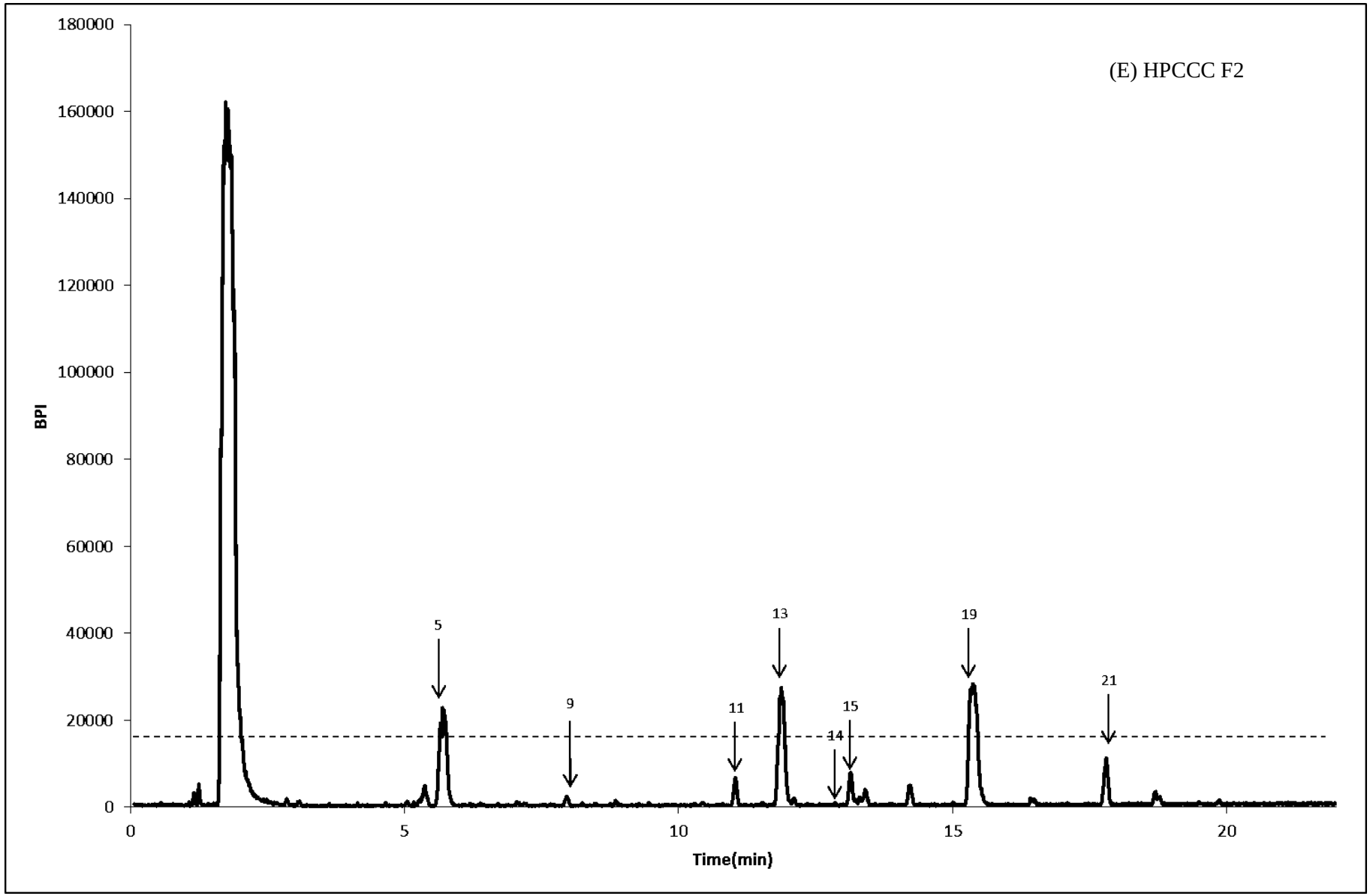
Figure S9. Estrogen receptor characterisation of the MCF-7 BUS cell line. Western Blot analysis reveals the presence of ERα and ERβ in the MCF-7 BUS breast cancer cell line. - indicates the negative control for the steroid receptor (untransfected COS-1 cells). MCF-7 BUS refers to the MCF-7 BUS lysate used for MCF-7 BUS estrogen receptor characterisation.











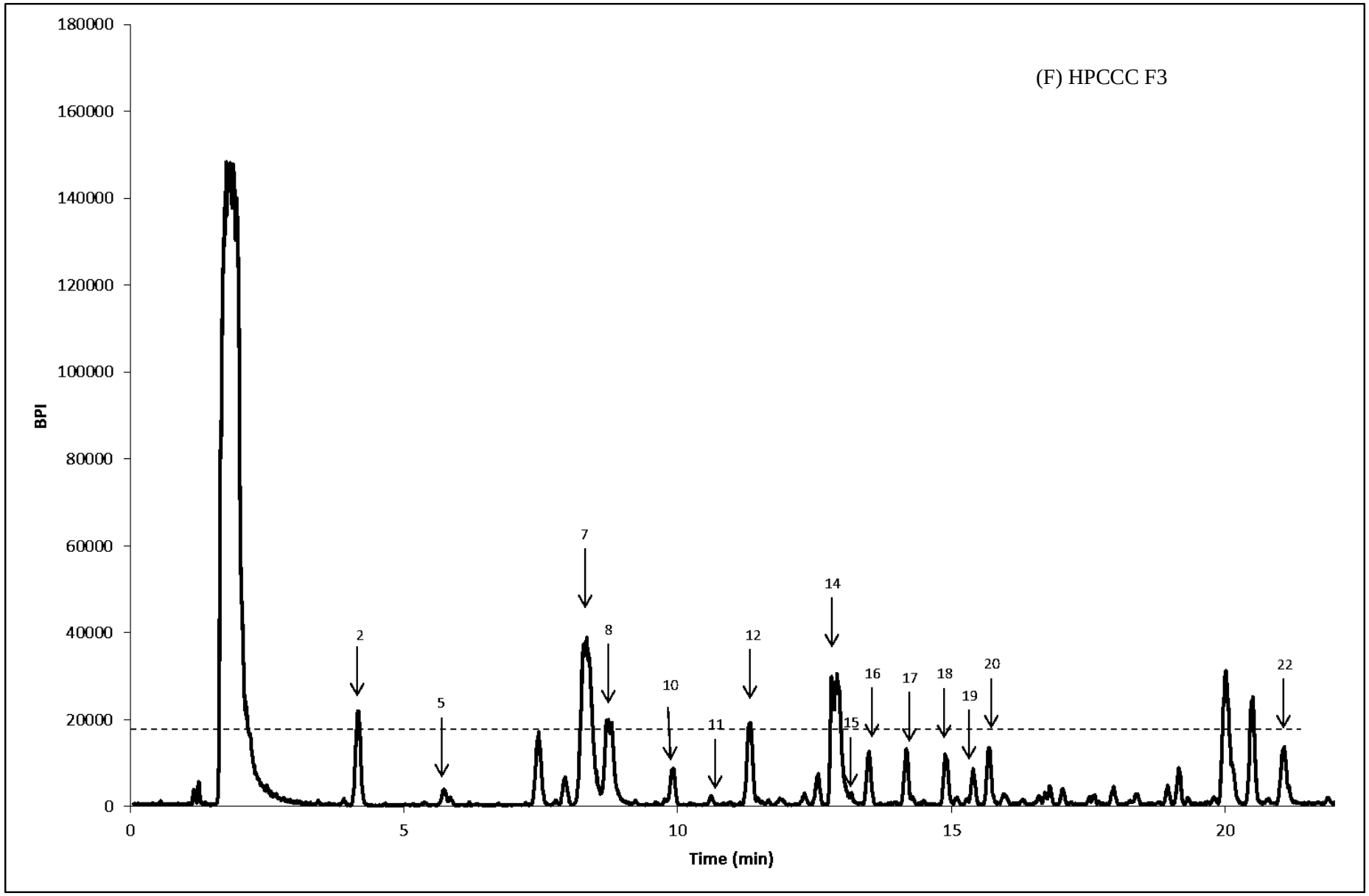


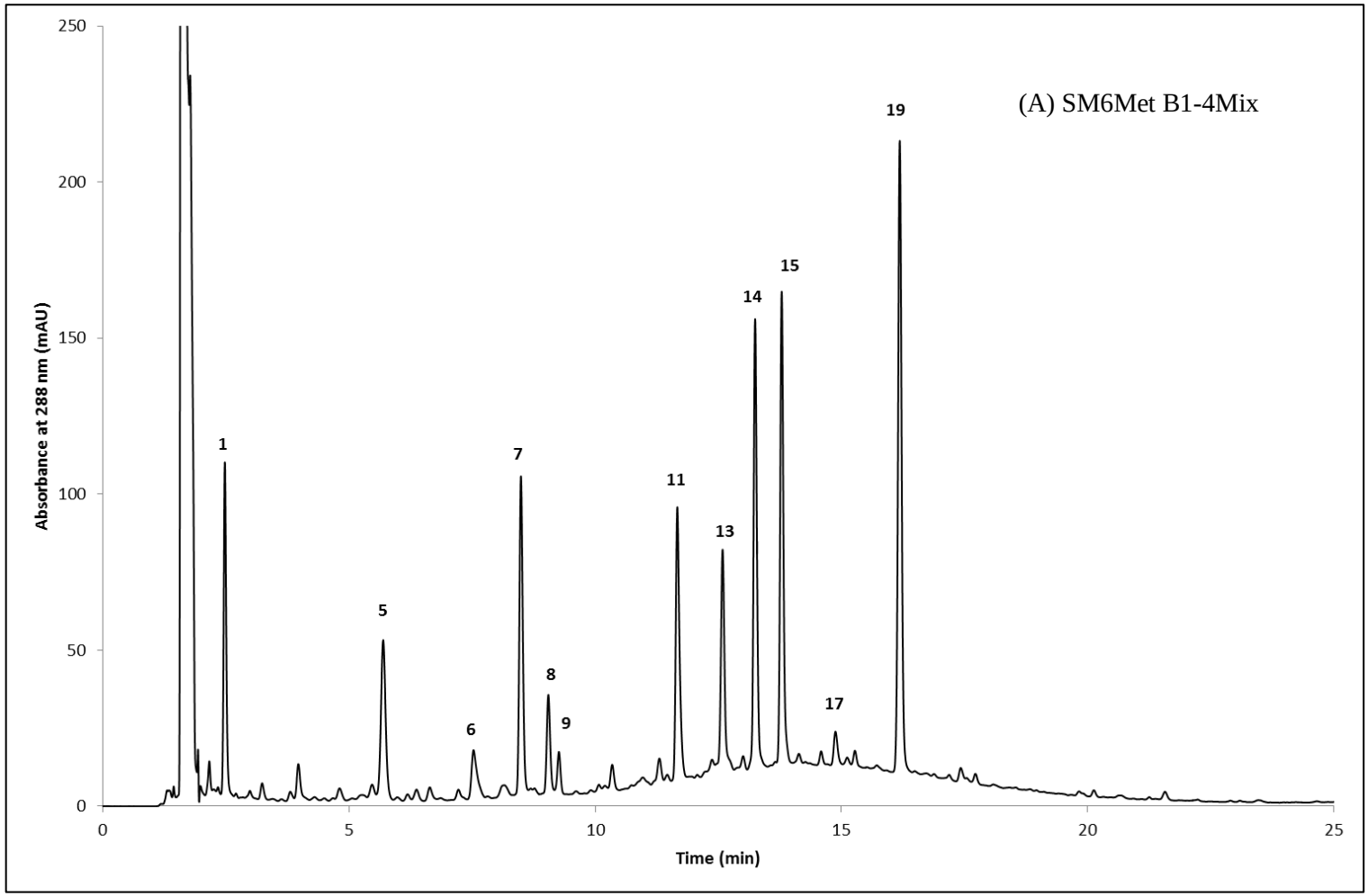
Figure S10A-F. LC-MS/MS analysis (graphs in base peak intensity, BPI) of SM6Met B1-4Mix (A) and fractions (NPF (B), PF (C), HPCCC F1 (D), HPCCC F2 (E), and HPCCC F3 (F)) obtained during activity-guided fractionation. Numbers correspond with Table S3. The initial peak in all samples was due to ascorbic acid added to prevent oxidation and not taken into account when numbering peaks. Peaks above the dotted lines are considered to be major peaks, whilst peaks below the dotted lines are considered to be minor peaks as indicated in Table S3.

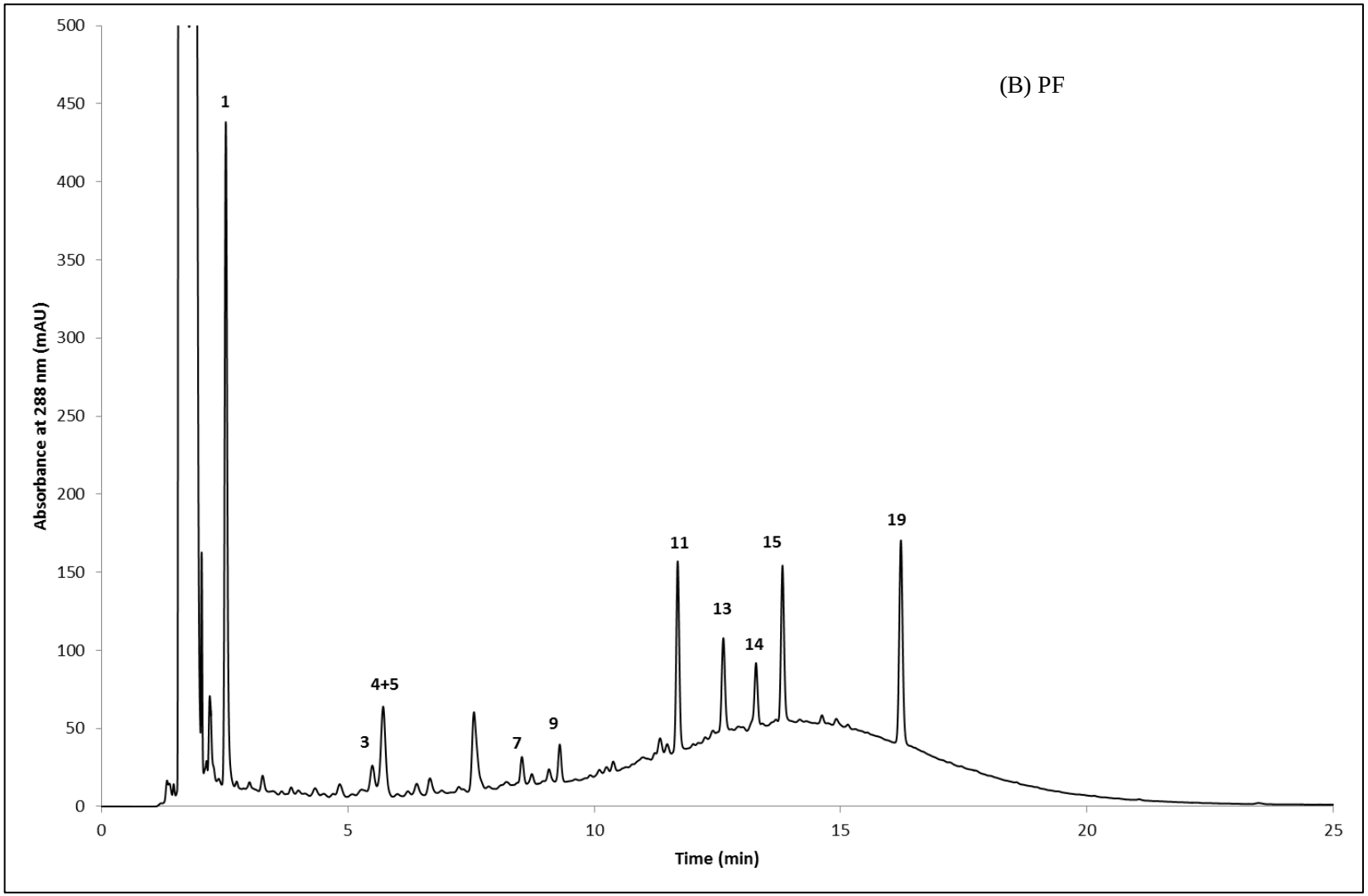
Table S3. LC-MS/MS data of compounds present in SM6Met B1-4Mix and subsequent fractions obtained during activity-guided fractionation.

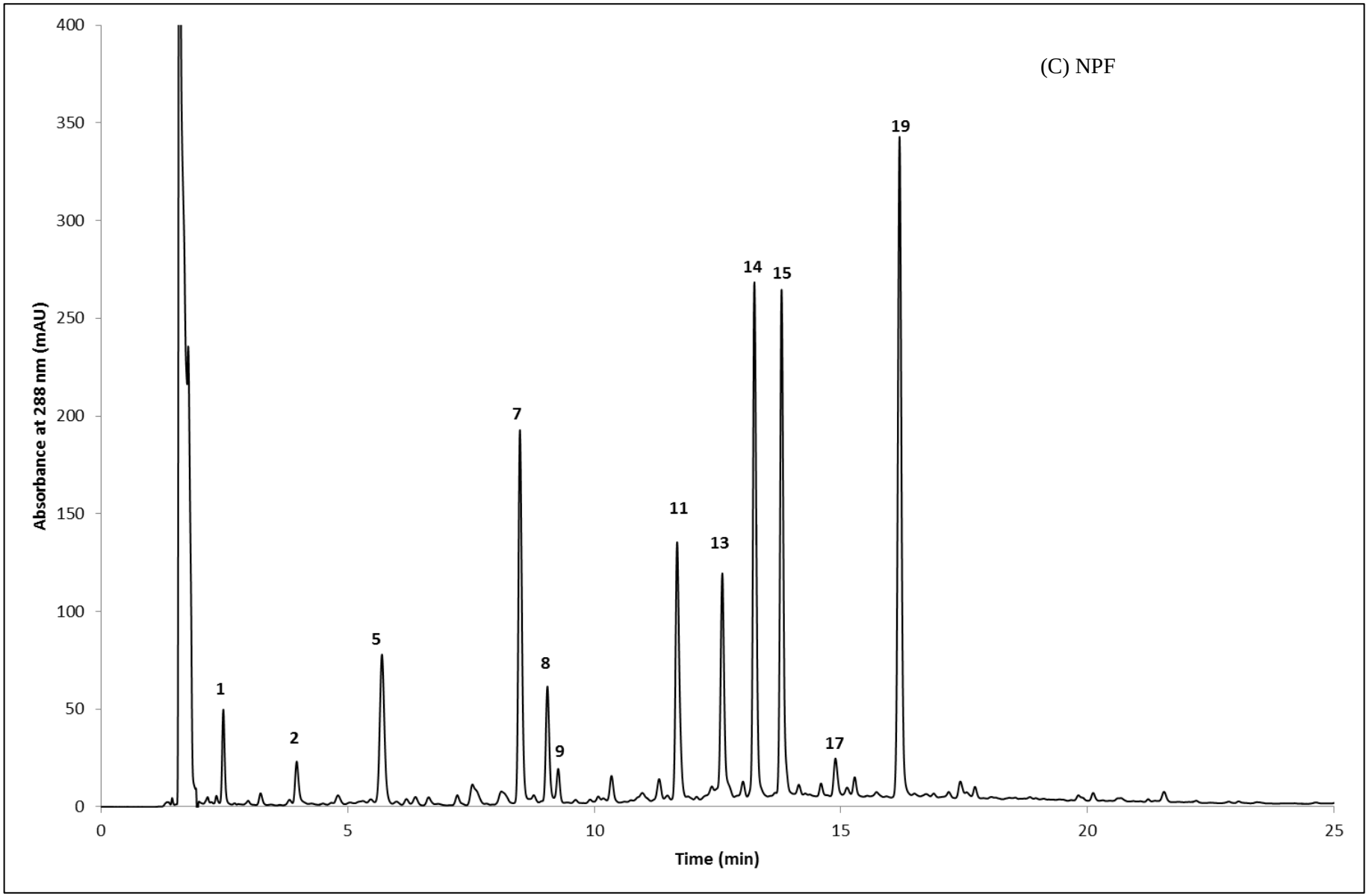
Nr	RT (MS)[a]	UV-Vis (λ_{max})	$[M-H]^-$	Fragment ions	Compound	Compound class	Notes[b]	SM6Met B1-4Mix	SM6Met PF	SM6Met NPF	HPCCC F1	HPCCC F2	HPCCC F3
1	2.7	293	569*[c]	479, 449, 317, 287*	Iriflophenone-di-*O*,*C*-hexoside	Benzophenone	[72]	x[d]	**X**	x	x		
2	4.1	259, 293	153*[c]	No data	Protocatechuic acid	Hydroxybenzoic acid	ID	x		x			**X**
3	5.6	286	611*	491, 431, 401, 371*	(*S*)-Eriodictyol-di-*C*-hexoside	Flavanone	[72]	x	x	x	x		
4	5.7	286	611*	491, 431, 401, 371*	(*R*)-Eriodictyol-di-*C*-hexoside	Flavanone	[72]	x	x	x	x		
5	5.8	294	407*	317, 287*, 257, 245, 215, 201, 193, 165, 125	Iriflophenone-3-*C*-glucoside	Benzophenone	[72]	**X**[e]	x	**X**		**X**	x
6	7.3	285	595*	475, 415, 385*, 355	Naringenin-di-*C*-hexoside	Flavanone	[72]	x	x	x	x		
7	8.4	239, 257, 317, 366	421*	331, 301*, 271, 259	Mangiferin	Xanthones	[72]	**X**	x	**X**			**X**
8	8.8	240, 255, 316, 365	421*	331, 301*, 271, 259	Isomangiferin	Xanthones	[72]	**X**	x	**X**			**X**
9	9.0	231, 270, 330	593*	503, 473*, 383, 353	Apigenin-6,8-di-*C*-glucoside (vicenin-2)	Flavone	[72]	x	x	x	**X**	x	
10	10.1	281	449*	287, 151*, 135	Eriodictyol-*O*-glucoside	Flavanone	[72]	x		x			x
11	11.2	285	613*	475, 433, 403, 373*, 361, 331, 209	3-Hydroxyphloretin-3',5'-di-*C*-hexoside	Dihydrochalcone	[72]	**X**	**X**	**X**	**X**	x	x
12	11.3	309	163*	No data	*p*-Coumaric acid	Hydroxycinnamic acid	ID	x	x	x			**X**
13	12.0	283	595*	287, 151*	Eriocitrin (eriodictyol-7-*O*-rutinoside)	Flavanone	[72]	**X**	x	**X**	**X**	**X**	
14	13.0	265, 348	593*	285	Scolymoside (luteolin-7-*O*-rutinoside)	Flavone	[72]	**X**	**X**	**X**		x	**X**

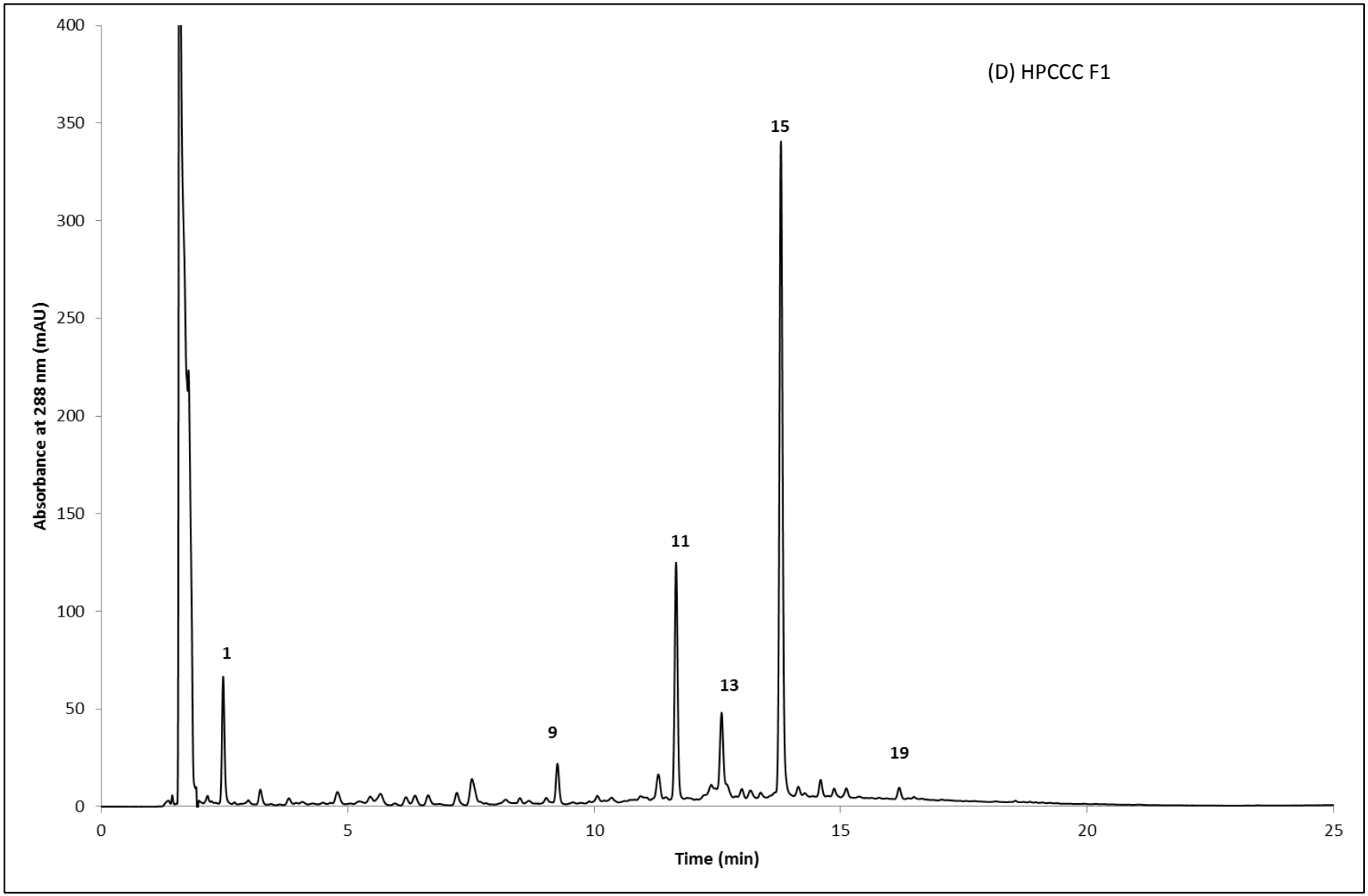
15	13.3	284	597*	459, 417, 387, 357*, 345, 315	Phloretin-3',5'-di-*C*-glucoside	Dihydrochalcone	[72]	**X**	**X**	**X**	**X**	x	x
16	13.6	254, 265, 348	447*	285	Luteolin-*O*-hexoside	Flavone	NC	x		x			x
17	14.3	282	579*	271*, 151	Naringenin-*O*-dihexoside	Flavanone	[72]	x	x	x			x
18	15.0	266, 337	577*	269	Apigenin-*O*-rutinoside/neohesperidoside	Flavone	NC	x	x	x			x
19	15.5	283	609*	301	Hesperidin (hesperitin-7-*O*-rutinoside)	Flavanone	[72]	**X**	**X**	**X**	x	**X**	x
20	15.8	252, 265, 346	607*	299*, 284	Chrysoeriol-*O*-rutinoside/neohesperidoside	Flavone	NC	x	x	x			x
21	18.0	259, 375	609*	301	Quercetin-*O*-rutinoside/neohesperidoside	Flavonol	NC	x				x	
22	21.0	252, 265, 348	285*	No data	Luteolin	Flavone	ID	x		x			x

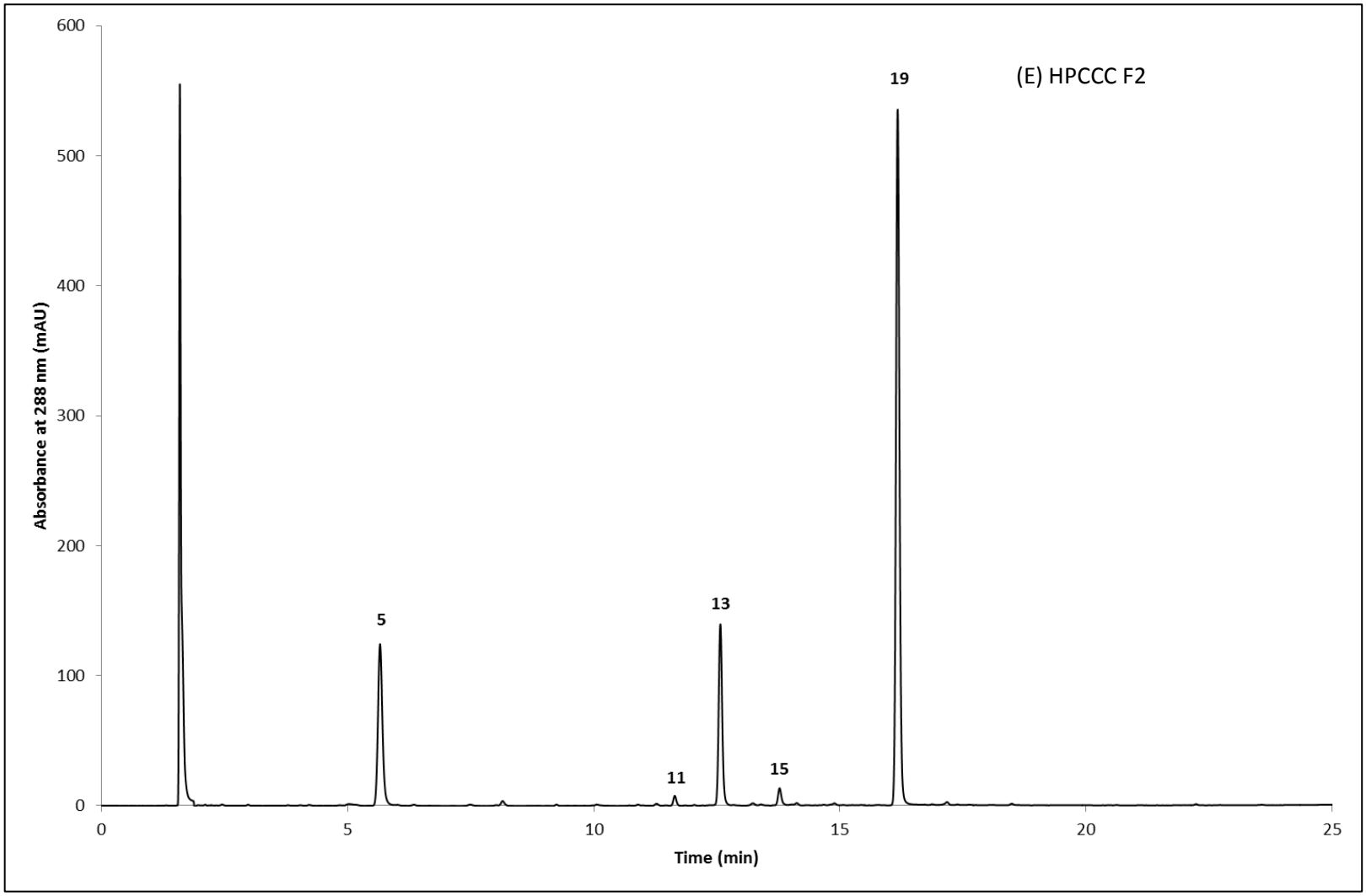
[a] indicates the polyphenol retention time
[b] MS data from literature used to tentatively identify compounds
[c]* indicates the deprotonated molecular ion species in the case of $[M-H]^-$ and in the case of the fragment ions it indicates the major daughter ion
[d]Minor polyphenols in extract are represented by "x"
[e]Major polyphenols in extract are represented by "X''
ID refers to the fact that the compound was identified by means of an authentic reference standard
NC refers to the presence of a compound not identified before and further structure elucidation is required











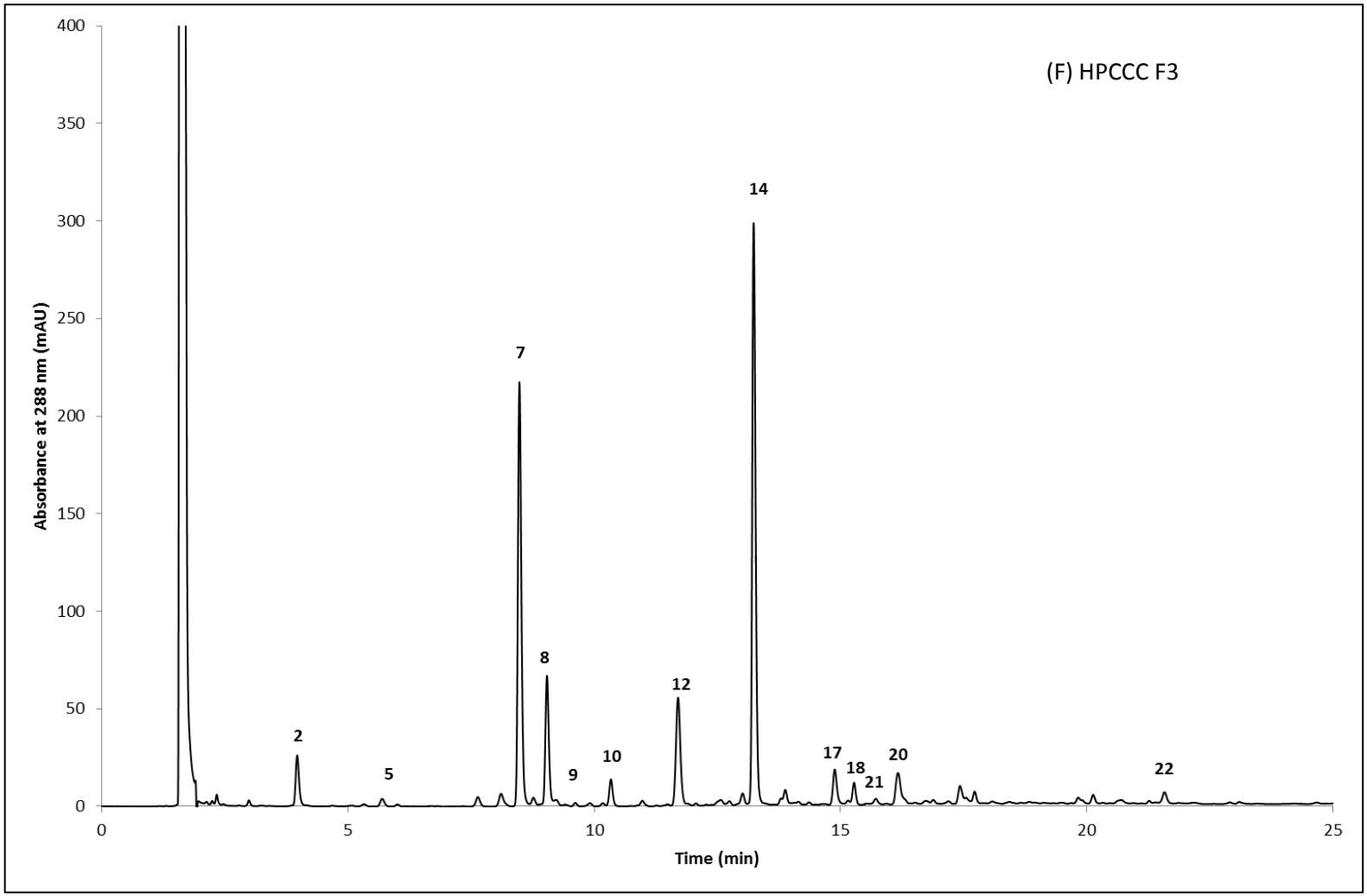


Figure S11A-F. HPLC-DAD chromatograms of SM6Met B1-4Mix (A) and fractions obtained during fractionation (PF (B), NPF (C), HPCCCF1 (D), HPCCCF2 (E), HPCCC F3 (F)). Numbers correspond with Table S3. The first peak in all samples was due to ascorbic acid added to prevent oxidation and was not included in the numbering of peak.

Chapter 4
Intestinal absorption of *Cyclopia subternata* extract, SM6Met

4.1 Introduction

Development of an oral nutraceutical would require screening for absorption into the human body. Intestinal absorption of bioactive polyphenols in plant extracts would be crucial for systemic availability and exertion of desired therapeutic effects [1-3]. Even though the process of intestinal absorption can be transcellular, via passive or active transport or paracellular, due to movement through tight junctions, the most common route for drugs is via a passive transcellular process [4]. Furthermore, pharmacokinetic studies have shown that this process may occur along the entire length of the intestine [5], and that the transport and distribution of most drugs are via passive diffusion, which depends on compound lipophilicity [6]. Also, literature suggests that for a compound to permeate the intestinal barrier, it needs to comply with a set of rules known as the Lipinski "Rule-of-5", in addition, unless actively transported, the compound needs to possess a polar surface area (PSA) less than 140$Å^2$, and less than 10 rotatable bonds (RB) [7,8]. Although several *in vivo* and *in vitro* models for prediction of human intestinal absorption have been used in recent years, these methods are labour intensive and give variable results [2]. The use HT methods such as QSPR determination methods [2], has become increasingly popular in modern science, of which physicochemical profiling is most commonly used [9].

The *Cyclopia subternata* extract, SM6Met, displays desirable attributes for the development of a phytoestrogenic nutraceutical to treat menopausal symptoms [10-13]. Thus investigation of the intestinal absorption of SM6Met is vital, as intestinal absorption serves as a preliminary qualification for the polyphenols within the extract to become part of the general systemic circulation and thus to exert a therapeutic effect [1]. No absorption studies have been performed on the SM6Met extract; however, research has been performed on other plant extracts and on some of the major polyphenols present in the SM6Met extract. For example, a study investigating the oral ingestion of a *C. genistoides* extract, with known mangiferin and hesperidin content, by pigs indicated that the xanthones, mangiferin and isomangiferin were not detected in plasma, but that their corresponding aglycone, norathyriol, was detected [14]. Furthermore, neither the flavanone,

hesperidin, nor any of its metabolites, were detected in the plasma, although several biotransformed metabolites of both mangiferin and hesperidin were detected in the urine [14]. In a separate study, absorption of an aspalathin-rich rooibos extract revealed no detection of aspalathin, a dihydrochalcone *C*-glucoside, in pig plasma, however, aspalathin and five dihydrochalcone metabolites were identified in urine, indicating the involvement of phase II enzyme metabolism [15]. Also, in a human study, aspalathin was detected in trace amounts in urine, however, no aspalathin was detected in the plasma [16]. In contrast, in an absorption study in rats, the dihydrochalcone aglycone, phloretin, was detected in rat plasma, mainly in conjugated form, although some unconjugated phloretin was also detected [17]. Furthermore, luteolin or luteolin-7-*O*-β-glucoside administered to rats via gastric intubation resulted in free luteolin and conjugates of luteolin in the plasma, but no luteolin-7-*O*-β-glucoside, suggesting hydrolysis and absorption of free luteolin [18]. Literature suggests that the hydrolysis of these glycoside residues arises to ensure permeability of the aglycone for optimal absorption [19]. *C*-glycosides are less likely to be hydrolysed than *O*-glycosides as the C-C bond confers acid stability [20]. They are, however, in some cases, still hydrolysed in the colon by bacterial *C*-glycosyl cleavage enzymes released intestinal flora, as in the case of mangiferin hydrolysis [21].

Whereas previous studies [14,15,17,18] showing absorption of some of the polyphenols present in SM6Met, accounted not only for intestinal absorption, degradation and/or modification by intestinal microflora and further hepatic metabolism, the current study focused only on intestinal absorption. Specifically, the movement of SM6Met polyphenols across the porcine small and large intestine was investigated using a flow-through diffusion system with collected fractions analysed using LC-MS/MS. In addition, physicochemical profiling as method for prediction of absorption of the individual polyphenols in SM6Met, was also performed using three open source programs.

4.2 Materials and methods

4.2.1 Tissue specimens

Tissue specimens were obtained from a local abattoir (Winelands Pork (PTY) LTD, Western Cape, South Africa). Small intestine (duodenum), ca. 10 cm after the stomach, and large intestine (colon), ca. 60 cm before the anus, were excised at a size of 30 cm each, from one pig. The specimens were then carefully rinsed with PBS (pH 7.4) at 20°C where after excess fat was carefully removed using a scalpel, without disturbing the integrity of the epithelial surface. Tissue disks were made from the top half of each tissue type, and were then snap frozen and stored at -80°C until used.

4.2.2 Sample preparation

SM6Met was made up to a final concentration of 244 mg/mL using PBS (pH 7.4) with ascorbic acid at a final concentration 9.1 mg/mL. The sample was then centrifuged at 5000 RPM (2991 x g) for 1 min to remove any insoluble material and the supernatant used in the permeability experiments. In addition, the supernatant used in each permeability experiment served as a positive control in LC-MS/MS analysis of fractions obtained.

4.2.3 Permeability experiments

The *in vitro* technique used for the analysis of diffusion of SM6Met polyphenols through the small and large intestine was the flow-through diffusion system (ISCO Retriever IV, ISCO®. USA), as adapted from Van Zyl *et al.* [22]. Tissue specimens were thawed at room temperature in PBS (pH 7.4) and then equilibrated for 10 min in the aforementioned PBS, where after they were investigated under the microscope for any potential tissue integrity disturbances before experiments were conducted. Specimens were then mounted (mucosal side facing the donor chamber) in flow-through diffusion cells (exposed area of 0.196 cm^2) as described by Van der Bijl and van Eyk [23]. Each experiment was performed concurrently on 7 tissue replicates. Before the experiment was started the mounted tissue specimens were allowed to equilibrate for a further 10 min in PBS (21°C or 37°C, pH 7.4, containing 9.1 mg/mL ascorbic acid) in the acceptor and donor compartments of each

diffusion cell. Thereafter the PBS was removed from the cells and replaced with 500 uL of SM6Met at a concentration of 244 mg/mL. PBS, containing 9.1 mg/mL (21°C or 37°C) was pumped through the acceptor compartments at a rate of 1.5 mL/h and fractions collected by means of a fraction collector (IPC High Precision Multichanger Dispenser ISMATEC, IDEX Health & Science®, Germany) at 2 h intervals for 24 h. The study was performed under sink conditions, i.e. at the end of each run the concentration of SM6Met in the acceptor chamber never exceeded 10 % of that in the donor chamber.

4.2.4 LC-MS/MS analyses

Analyses of collected fractions and 'untreated' SM6Met were performed on a Waters Acquity UPLC system containing an in-line degasser, binary pump, column oven and PDA detector (Waters, Milford, MA, USA). This system was coupled to a Synapt G2 QTOF mass spectrometer (Waters) containing an ESI source. Ascorbic acid was added at a concentration of 9.1 mg/mL. The samples were then filtered using Millex-GV syringe filters (Millipore) with a 0.22 µm pore size. The HPLC method of De Beer et al. [24] was adapted for UPLC use. Separation was achieved on a Gemini-NX C18 (150 × 2 mm; 3 µm; 110 Å) column with 2 % acetic acid (A) and acetonitrile (B) as mobile phases. A flow rate of 0.320 mL/min for 0-20.1 min and 0.300 mL/min for 20.1-21.1 min was used with the following mobile phase gradient: 0-0.5 min (8.5 % B), 0.5-20 min (8.5-34.5 % B), 20-20.10 min (34.5-90 % B), 20.1-21 min (90 % B), and 21-21.1 (90-8.5 % B). An injection volume of 5 µL was used for all samples with the eluant being split 1:1 before introduction into the ionization source. MS results were acquired using negative ionization in MS^E mode. A collision energy ramp from 25 to 60 V was used, whereas for MS/MS data a collision energy of 30 V was used. The other MS parameters were the same as in De Beer *et al*. [24]: capillary voltage, 2.5 kV, cone voltage 15 V, desolvation temp 275°C, source temp 120°C and nitrogen flow rate 650 L/h. Peaks were identified by means of comparing LC-MS spectra and relative retention times with data in De Beer *et al*. [24]. Peak areas of targeted compounds were acquired by means of MassLynx v4.1 software

(Waters, USA) (manual integration) and TargetLynx (automated integration). Compounds were quantified in terms of peak area as no calibration standards were employed.

4.2.5 Computational tools

Three open source software programs were used to calculate the physicochemical properties of the individual polyphenols in SM6Met as a prediction tool for absorption of polyphenols. These programs were Osiris Property Explorer [25], ACD/Labs and ChemAxon [26].

4.2.6 Data analysis

Absolute peak areas were obtained for SM6Met ($\mathbf{A_0}$) and fractions ($\mathbf{A_2}$, $\mathbf{A_4}$, $\mathbf{A_6}$, etc) collected every 2 h for 24 h (Fig. S1-3, Table S1). The cumulative quantity of a compound that diffused at a specific time point ($\mathbf{t_n}$) was calculated as the sum of the individual peak areas of the compound at the time points leading up to the specific time point of interest: cumulative peak area ($\mathbf{A_{Cn}}$)

$$\mathbf{A_{Cn} = A_2 + A_4 + A_6 + \ldots A_n}$$

For example the cumulative peak area of a specific compound at 24 h ($\mathbf{A_{C24}}$), divided by the absolute peak of the same compound ($\mathbf{A_0}$), was used to calculate the percentage diffusion, i.e. the percentage of the parent compound that crossed the intestinal mucosa at t = 24:

$$\mathbf{\% \ diffusion = A_{C24} / A_0 \times 100}$$

Flux values (**J**) were calculated for each compound, taking into account the quantity (ng) of the compound (**Q**) that crossed the intestinal mucosa during the time (**t**) of exposure (min), and the intestinal area exposed (**A**) (cm^2). For the present investigation the area of exposure was 0.196 cm^2:

$$\mathbf{J = (dQ/dt \times 1/A)}$$

Q of each compound at **t** = n was calculated taking into account the composition of SM6Met (as determined in Chapter 3), the volume of SM6Met solution (500 uL of 244 mg/mL solution) added to the chamber, and the percentage diffusion of the compound at t = n.

The apparent permeability coefficients were calculated as follows [22]:

$$P_{app}=J/C_o$$

C_o is the initial concentration of the compound of interest (mg/mL), i.e. concentration in the starting mixture of SM6Met (244 mg/mL).

4.2.7 Data manipulation and statistical analysis

GraphPad Prism® version 5 (GraphPad Software, San Diego, CA) was used for graphical representation and statistical analysis of experimental data.

4.3 Results

Identification of a possible marker compound or compounds for correlation with estrogenic activity forms an integral part of the development of an oral phytoestrogenic nutraceutical (Chapter 3). Investigation into whether the compounds of interest will be absorbed and to what extent absorption will take place, is also crucial, due to the fact that for any nutraceutical to have an anticipated therapeutic effect, its active constituents need to enter the host bloodstream and reach target organs in adequate concentrations [27]. For this to take place compounds need to cross the lining of the gut after ingestion. Studies have shown that absorption of polyphenols, whether via passive or active transport [28], may take place in either the small intestine or large intestine depending on the compound investigated [14,18,29-32]. As mentioned earlier, absorption of compounds depend on the physicochemical properties of compounds such as, amongst others, molecular weight (MW), lipophilicity, PSA and the RB number [33], but further research shows that it also depends on physiological factors such as regional permeability differences, pH and luminal and mucosal enzymes [34]. We thus decided to perform a preliminary study focussing on only the absorption of polyphenols present in a *Cyclopia subternata* extract, SM6Met [12]. The flow-through diffusion apparatus used in the current *ex vivo* study has been previously used for absorption studies investigating absorption of drugs across bronchial tissue [22]. As absorption may take place in either the small or large intestine we investigated both tissues. Fractions obtained from the flow-

through diffusion assay was analysed using LC-MS/MS for detection of polyphenols originating from SM6Met.

4.3.1 Optimisation of the flow-through diffusion assay for use as an accurate model for intestinal absorption of polyphenolic compounds

A preliminary assay investigating diffusion through the small intestine was performed at room temperature (21°C). Although not physiologically relevant, low temperature was considered to minimise phenolic degradation as these type of compounds are instable at a high pH, such as pH 7.4 [35-37]. At this temperature the polyphenolic compounds where detected only after 20 h (Fig. 1A, Fig. S1). Thereafter the assay temperature was increased to the physiologically relevant 37°C (Fig. 1B, Fig. S2), resulting in the detection of compounds within 6-10 h, which is in agreement with previous findings of studies in rats [17,18,38-40]. Also, isomangiferin and 3-hydroxyphloretin-3',5'-di-*C*-hexoside were detected when the assay was performed at 37°C, but not at 21°C. These findings suggested that 37°C, rather than 21°C, should be used for further investigation.

The temperature-'optimised' assay procedure was then duplicated for investigation of absorption by the large intestine, however, no polyphenols were detected within the 24 h experimental period (Fig. 2B, Fig.S3).

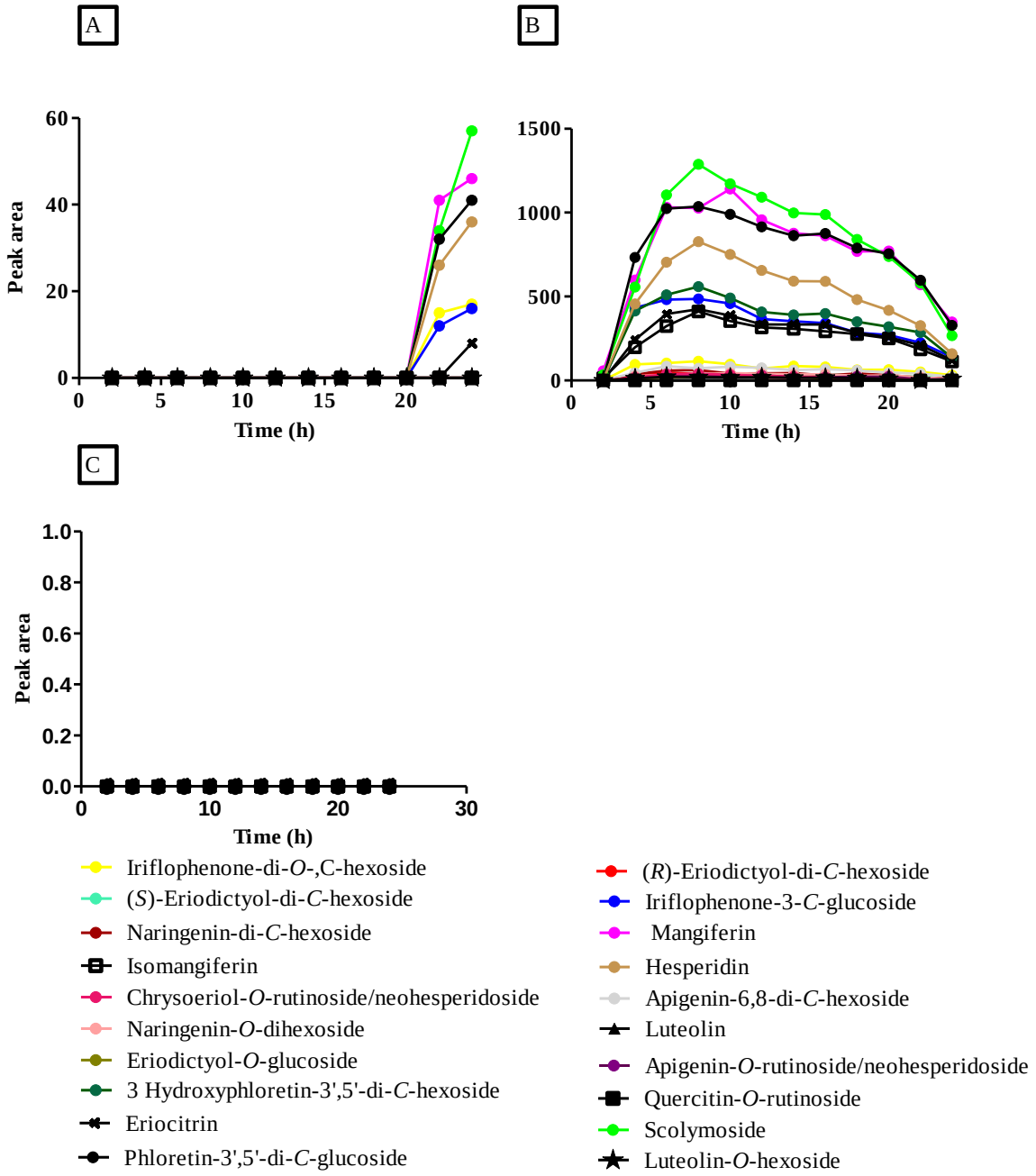


Figure 1 Optimisation of the flow-through diffusion assay for diffusion analysis of polyhenolic compounds in SM6Met across porcine small and large intestine. The permeability assay evaluating the diffusion of SM6Met polyphenols across porcine small intestine using the flow-through diffusion system [22] was performed at 21°C (A) and at 37°C (B) over 24 h. In (C) the temperature-'optimised' assay (37°) was used for evaluation of diffusion of SM6Met polyphenols across porcine large intestine. Fractions obtained from the flow-through diffusion assay were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. Values obtained were expressed as peak areas. Graphs displayed were obtained from one replicate per assay performed, and thus served as a representative graph for all other replicates in the assay.

As the time of diffusion of the polyphenols through the small intestinal mucosa obtained with the flow-through diffusion model correlated to some extent with previous studies in rats [17,18,38-40], and no permeation was observed through the large intestinal mucosa, differing from previous

studies [41-43], we decided to focus solely on the results obtained for the optimised procedure investigating the small intestine in the following section.

4.3.2 Detailed analysis of diffusion across small intestine at 37°C

4.3.2.1 Inter replicate variability

A single flow-through diffusion experiment was performed, which consisted of 7 replicates. Since one of the replicates leaked, LC-MS/MS analysis of the fractions collected from cells 2-7 was performed. An averaged peak area, calculated from the peak areas of the individual compounds, was used to plot diffusion over time in order to assess the replicates. A large degree of variation in absorption time and peak area was observed between replicates (Fig 2A-F). Replicates 2, 3, 4 and 7 (Fig. 2A-C&F) presented fairly similar plots, whereas replicates 5 and 6 (Fig. 2D&E) displayed substantially lower peak areas at corresponding time points for the 24 h period. Consequently, as replicates 5 and 6 were considered outliers, and their data were excluded from our more detailed analysis of polyphenol diffusion across the small intestine. Comparing the average peak area-time plots of the replicates, pre- and post-exclusion, showed similar trends, with slightly higher values post-exclusion of replicates 5 and 6 (Fig. 2G&H).

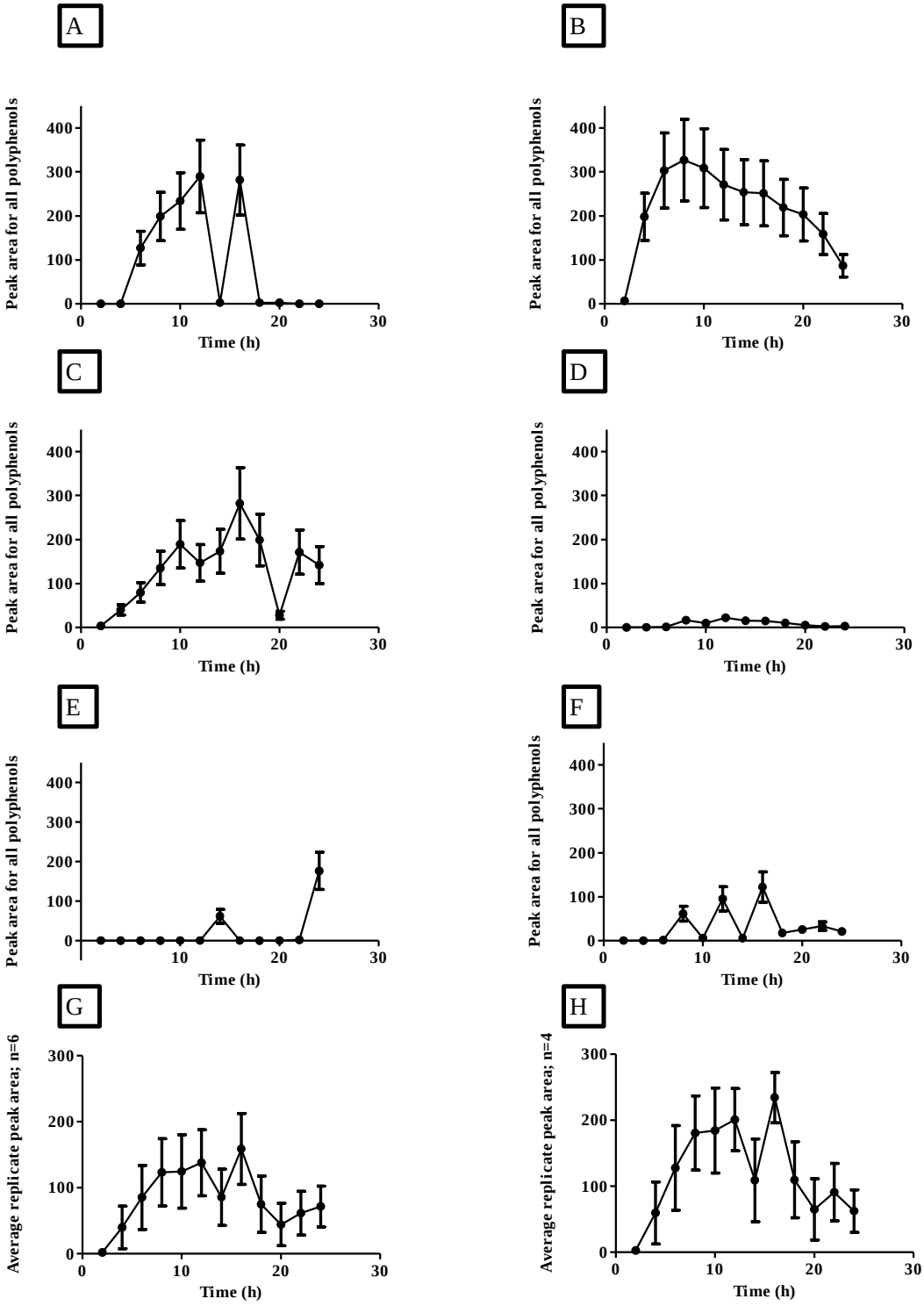


Figure 2 Evaluation of inter-replicate variability on the diffusion of all SM6Met polyphenols across porcine small intestine. The permeability assay was performed at 37°C over 24 h, using a flow-through diffusion system [22] with fractions collected at 2 h intervals. Fractions were analysed using LC-MS/MS to determine the presence and quantity of polyphenols originating from SM6Met. Graphs A-F represent the individual replicates obtained from the assay, with the average peak area for all compounds plotted over time: A) Replicate two; B) replicate three; C) replicate four; D) replicate five; E) replicate six; F) replicate seven. Graphs G and H represent the average of data shown in graphs A-F and graphs A-C; F (data of replicates five and six excluded), respectively.

4.3.2.2 Analysis of diffusion of individual polyphenols originating from SM6Met across porcine small intestine

As the peak area of a compound at a specific time takes into account only the amount of a polyphenol that has crossed over the mucosa during the preceding 2 h interval (Fig 1B), it does not reflect the total amount of polyphenol that has diffused through the mucosa up to that time point. Consequently, we decided to display our data in a manner that would reflect the cumulative diffusion of a specific polyphenol over time (Fig. S4-8). However, as previous studies have shown that absorption or diffusion of compounds are concentration driven [44-46], the initial concentration of each polyphenol in the SM6Met extract was taken into account by calculating the % of polyphenol that diffused across the intestinal lining (Fig. 3-7)

The cumulative peak area for the xanthones, mangiferin and isomangiferin, that diffused over 24 h (Fig. 3A, Fig. S4) in relation to their peak areas for the SM6Met solution in the donor chamber at t = 0, indicates a concentration dependent diffusion for the xanthones in this model. Slightly better diffusion was observed for mangiferin that isomangiferin with 7.6 ± 2.5 % of the initial amount of mangiferin (Fig. 3B&C) moving across the porcine intestine, compared to 6.5 ± 2.2 % for isomangiferin (Fig. 3D&E).

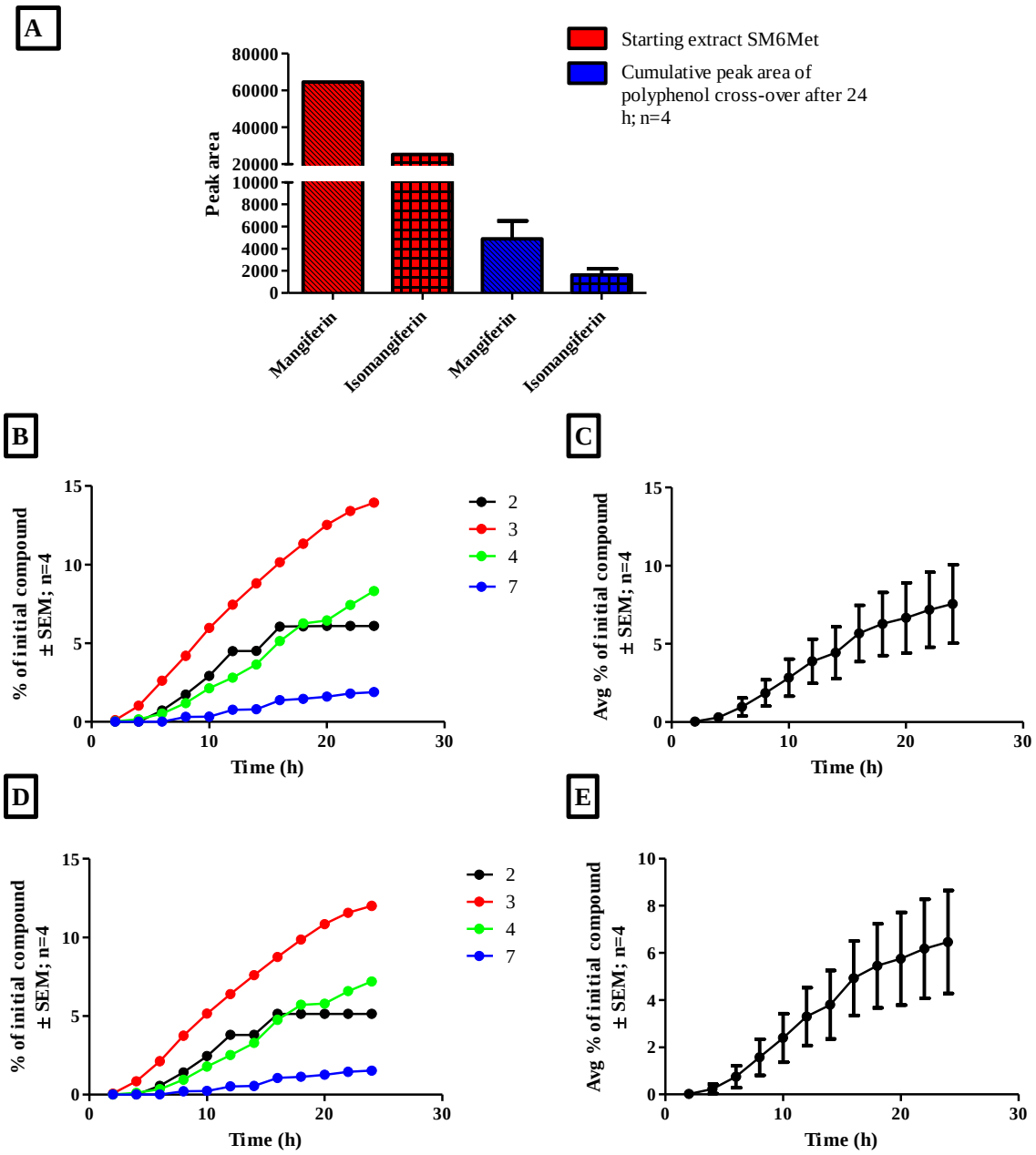
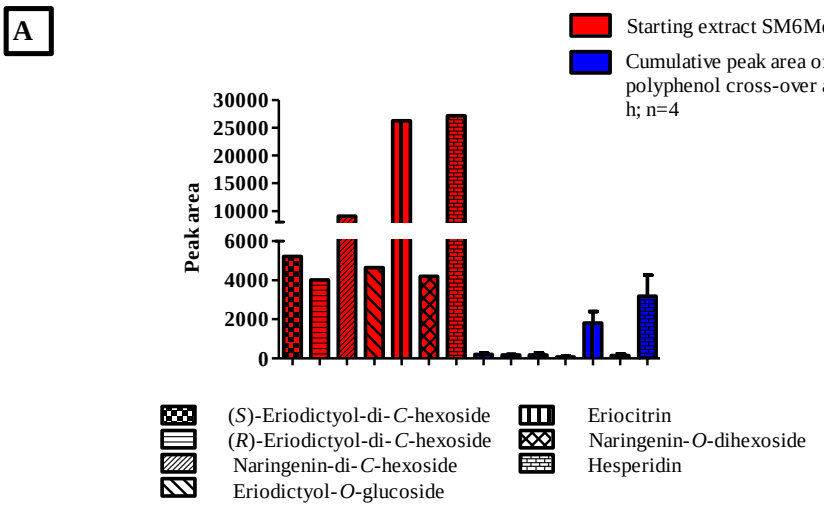


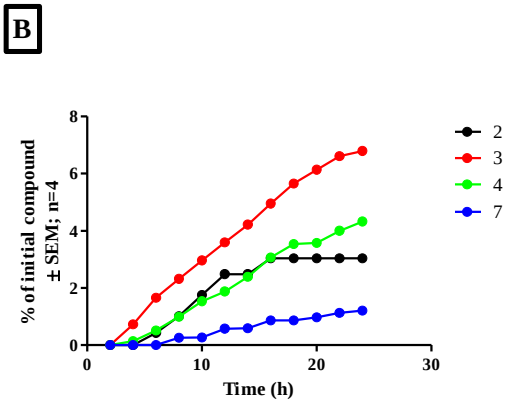
Figure 3 Diffusion analysis of the xanthones originating from SM6Met via porcine small intestine. The permeability assay was performed at 37°C, over 24 h, using a flow-through diffusion system [22] with fractions collected at 2 h intervals. Fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) Peak area values displayed in red for the individual polyphenols as present in SM6Met, and cumulative peak areas displayed in blue, portraying the amount of the polyphenols that permeated the small intestine over 24 h. Graphs B-E represent the cumulative peak area as a percentage of the polyphenol peak area in SM6Met at t = 0; B) individual replicate percentage diffusion of mangiferin; C) average replicate percentage diffusion of mangiferin; D) individual replicate percentage diffusion of isomangiferin; E) average replicate percentage diffusion of isomangiferin.

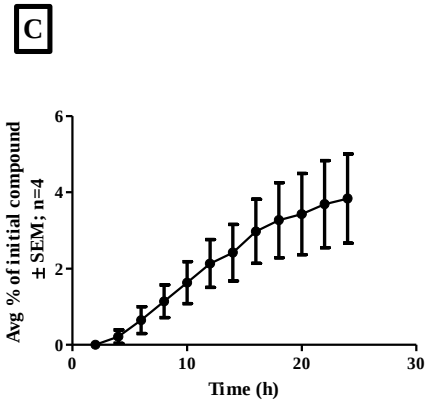
For the flavanones, the only two polyphenols crossing the small intestine in relatively high amounts were eriocitrin and hesperidin (Fig 4A, Fig. S5), however, this might again be due to a concentration dependent diffusion effect as the flavanones present at lower concentrations in the starting mixture diffused across the small intestine in lowered amounts. Thus, only 3.4 ± 1.8 %-4.1

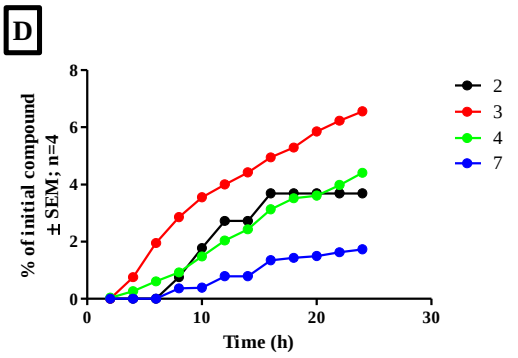
±1.0 % of the initial (*S*) and (*R*)-eriodictyol-di-*C*-hexoside and naringenin-*O*-dihexoside in SM6Met diffused across the porcine small intestine (Fig 4B-G). In contrast, 6.9 ± 2.3 %-11.7 ± 3.9 % of the initial amount of hesperidin and eriocitrin diffused across the small intestine by 24 h (Fig. 4H-K), while 1.5 ± 0.9 %-2.0 ± 1.0 % of the initial eriodictyol-*O*-glucoside and naringenin-di-*C*-hexoside diffused (Fig. 4L-O). These results suggest that when considering starting concentrations, the flavanones present in SM6Met diffuse across the porcine small intestine in the following order of increasing preferential diffusion: eriodictyol-*O*-glucoside, naringenin-di-*C*-hexoside, naringenin-*O*-dihexoside, (*S*) and (*R*)-eriodictyol-di-*C*-hexoside, eriocitrin, and hesperidin.

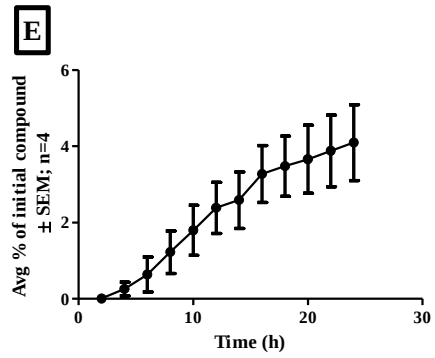
When considering the individual diffusion profiles of the compounds for each replicate, some trends were evident. The two major flavanones, hesperidin and eriocitrin, displaying the highest percentage diffusion, had similar diffusion profiles (Fig. 4H&J). A similar trend was also observed for the two polyphenols with the lowest percentage diffusion (Fig. 4L&N). In addition naringenin-di-*C*-hexoside showed similar replicate profiles to that of naringenin-*O*-dihexoside (Fig. 4F&N), whereas (*S*)- and (*R*)-eriodictyol-di-*C*-hexosides displayed similar profiles (Fig. 4B&D). Furthermore, the replicate profiles of the latter compounds were also similar to that of hesperidin and eriocitrin (Fig. 4B, D, H&J). These results suggest that the primary polyphenol structure rather than the sugar moiety or the position of the sugar moiety determined the absorption profile. Hesperetin, the aglycone of hesperidin, is similar in structure to eriodictyol, except for the methylated C3'-OH.

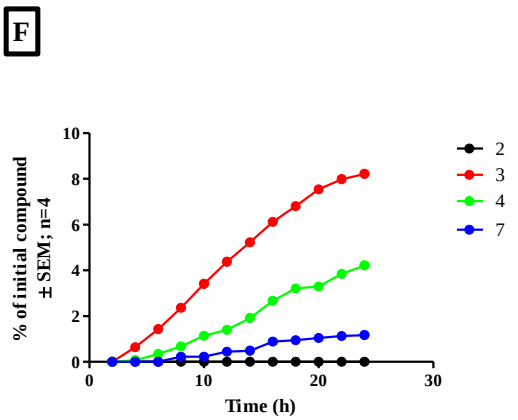


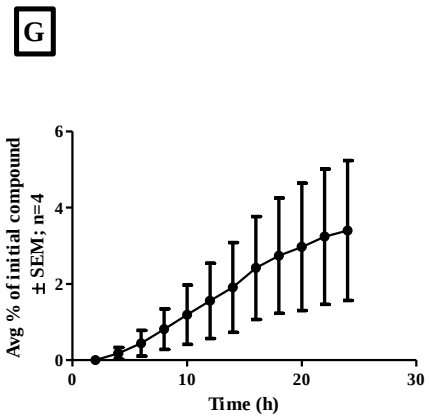












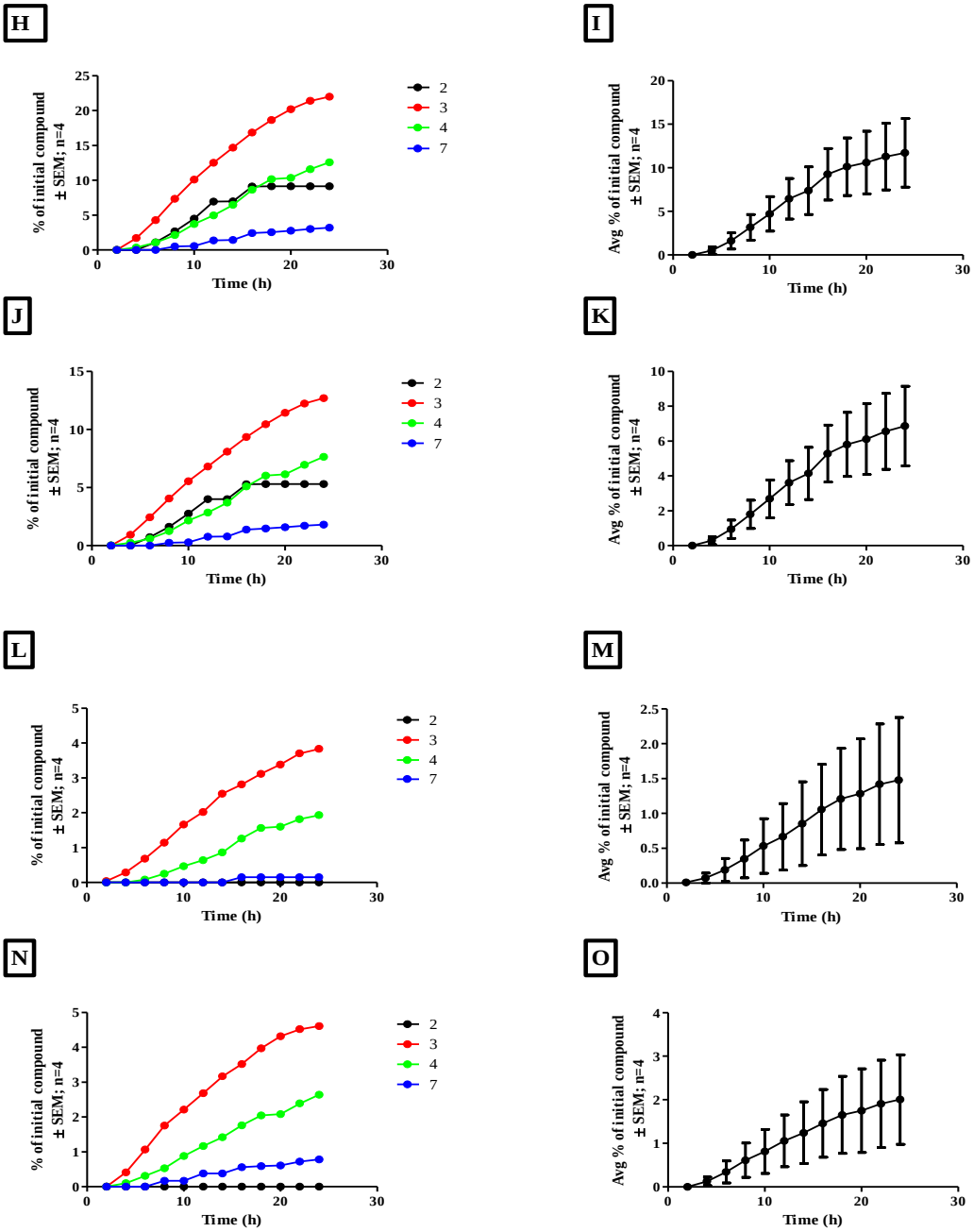


Figure 4 Diffusion analysis of the flavanones originating from SM6Met via porcine small intestine by means of comparing accumulated polyphenol small intestinal cross-over relative to its starting concentration. The permeability assay was performed at 37°C, over 24 h, using the flow-through diffusion system [22], with fractions collected at 2 h intervals. Fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) Peak area values displayed in red for the individual polyphenols as present in SM6Met, and cumulative peak areas displayed in blue, portraying the amount of the polyphenols that permeated the small intestine over 24 h.; Graphs B-O represent the cumulative peak area as a percentage of the polyphenol peak area in SM6Met at t = 0; B) individual replicate contribution of (*S*)-eriodictyol-di-*C*-hexoside; C) average replicate contribution of (*S*)-eriodictyol-di-*C*-hexoside; D) individual replicate contribution of (*R*)-eriodictyol-di-*C*-hexoside; E) average replicate contribution of (*R*)-eriodictyol-di-*C*-hexoside; F) individual replicate contribution of naringenin-*O*-dihexoside; G) average replicate contribution of naringenin-*O*-dihexoside; H) individual replicate contribution of hesperidin; I) average replicate contribution of hesperidin; J) individual replicate contribution of eriocitrin; K) average replicate contribution of eriocitrin; L) individual replicate contribution of eriodictyol-*O*-glucoside; M) average replicate contribution of eriodictyol-*O*-glucoside; N) individual replicate contribution of naringenin-di-*C*-hexoside; O) average replicate contribution of naringenin-di-*C*-hexoside

Considering the flavones, scolymoside and apigenin-6,8-di-*C*-glucoside were present in the highest amount in the starting mixture, SM6Met (Fig. 5A, Fig. S6), and they also showed also the highest diffusion levels across the small intestine after 24 h. Taking into account the initial concentration in the donor chamber, most of the flavones displayed a relatively low diffusion after 24 h, with percentages ranging from 1.6 ± 0.9% to 2.4 ± 0.8% for apigenin-*O*-rutinoside/neohesperidoside, chrysoeriol-*O*-rutinoside/neohesperidoside, luteolin-*O*-hexoside and apigenin-6,8-di-*C*-glucoside (Fig. 5C, G, I&K). In contrast, 11.5 ± 3.9% of the initial concentration of scolymoside diffused across the porcine small intestine within 24 h (Fig. 5E).

Analysis of variability introduced by each replicate revealed that scolymoside and apigenin-6,8-di-*C*-glucoside shared a similar replicate diffusion profile (Fig. 5D&J), whereas apigenin-*O*-rutinoside/neohesperidoside and chrysoeriol-*O*-rutinoside/neohesperidoside shared a similar profile (Fig. 5B&F). Luteolin-*O*-hexoside was the only flavone where only 2 out of the 4 replicates displayed diffusion across the porcine small intestinal mucosa (Fig. 5H).

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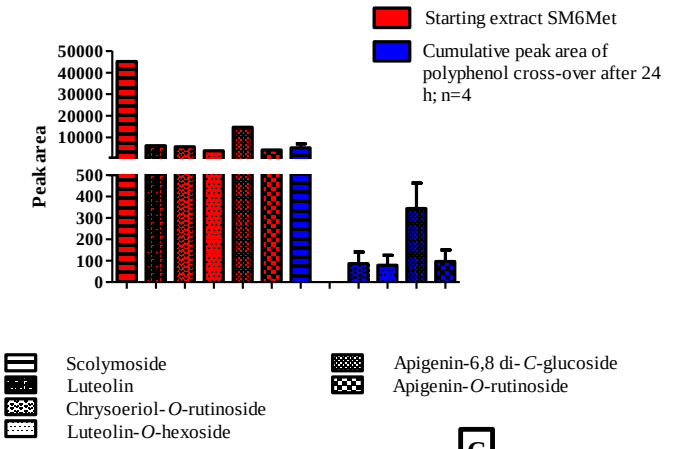

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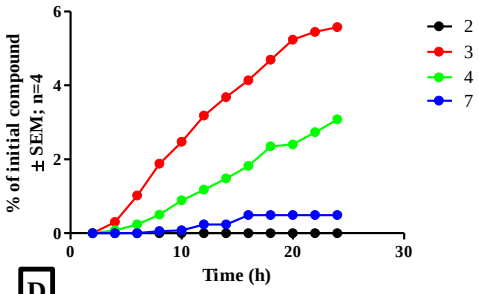

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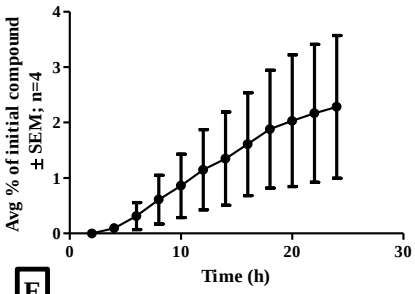

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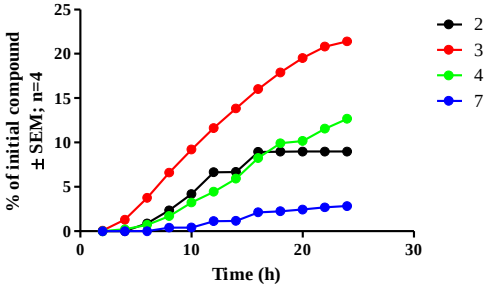

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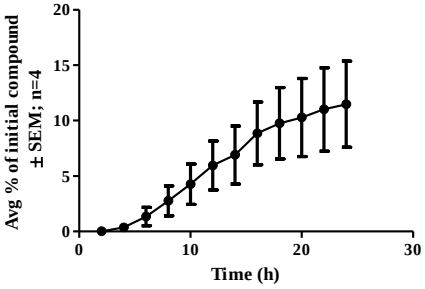

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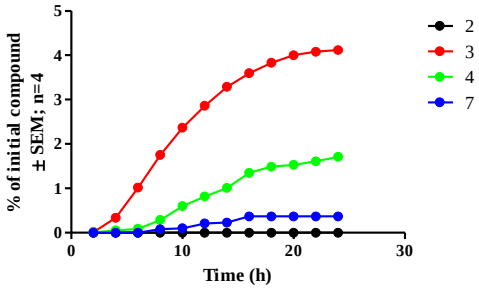

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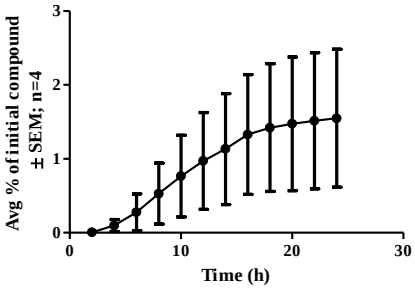

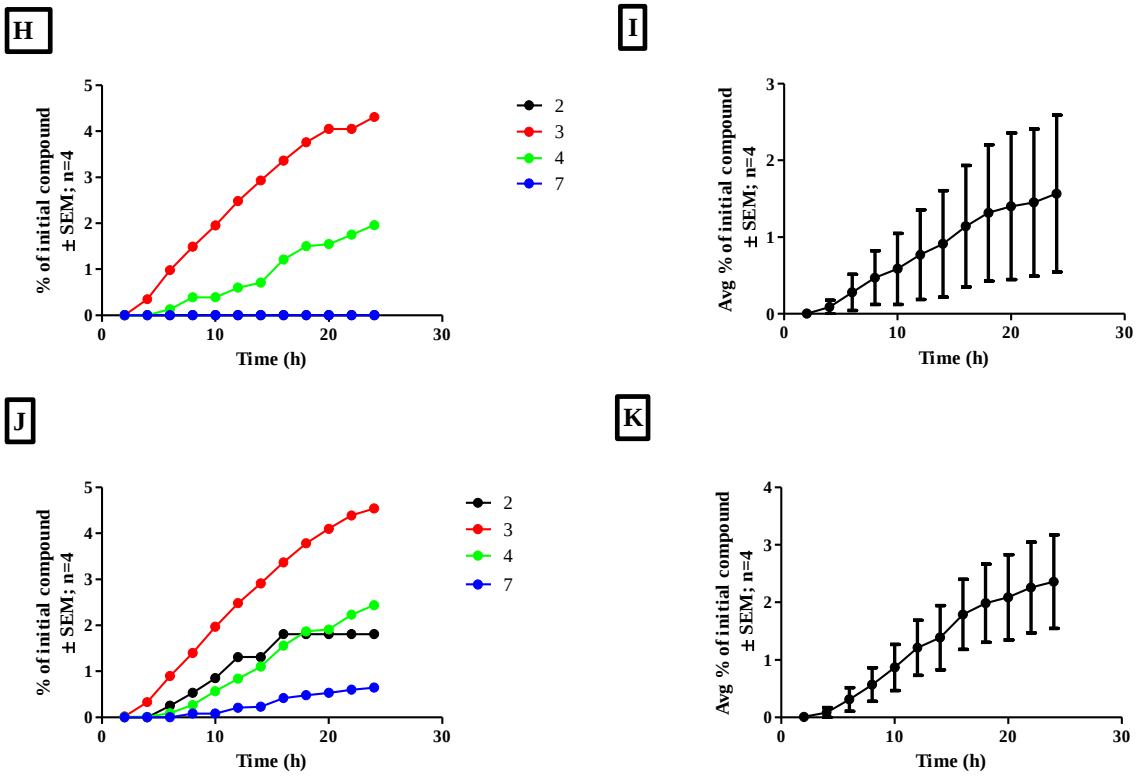

Figure 5 Diffusion analysis of the flavones originating from SM6Met via porcine small intestine by means of comparing accumulated polyphenol small intestinal cross-over relative to its starting concentration. The permeability assay was performed at 37°C, over 24 h, using the flow-through diffusion system [22], with fractions collected at 2 h intervals. Fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) Peak area values displayed in red for the individual polyphenols as present in SM6Met, and cumulative peak areas displayed in blue, portraying the amount of the polyphenols that permeated the small intestine over 24 h.; Graphs B-K represent the cumulative peak area as a percentage of the polyphenol peak area in SM6Met at t = 0; B) individual replicate contribution of apigenin-*O*-rutinoside; C) average replicate contribution of apigenin-*O*-rutinoside; D) individual replicate contribution of scolymoside; E) average replicate contribution of scolymoside; F) individual replicate contribution of chrysoeriol-*O*-rutinoside; G) average replicate contribution of chrysoeriol-*O*-rutinoside; H) individual replicate contribution of luteolin-*O*-hexoside; I) average replicate contribution of luteolin-*O*-hexoside; J) individual replicate contribution of apigenin-6,8-di-*C*-glucoside; K) average replicate contribution of apigenin-6,8-di-*C*-glucoside.

Investigation of the dihydrochalcones and benzophenones also revealed concentration dependent diffusion over the porcine small intestine (Fig. 6A & 7A, Fig. S7&8). The percentage of diffusion ranged between 7.1 ± 3.0% for iriflophenone-di-*O*,*C*-hexoside and 12.7 % ± 4.1% for phloretin-3',5'-di-*C*-β-glucoside (Fig 6C,E & Fig.7C&E).

A similar replicate diffusion profile was evident for the benzophenones and dihydrochalcones (Fig. 6B, D & Fig. 7B), with the exception of iriflophenone-di-*O*,*C*-hexoside (Fig. 7D). In the latter case replicate two displayed substantially higher percentage diffusion than replicates 3, 4 and 7.

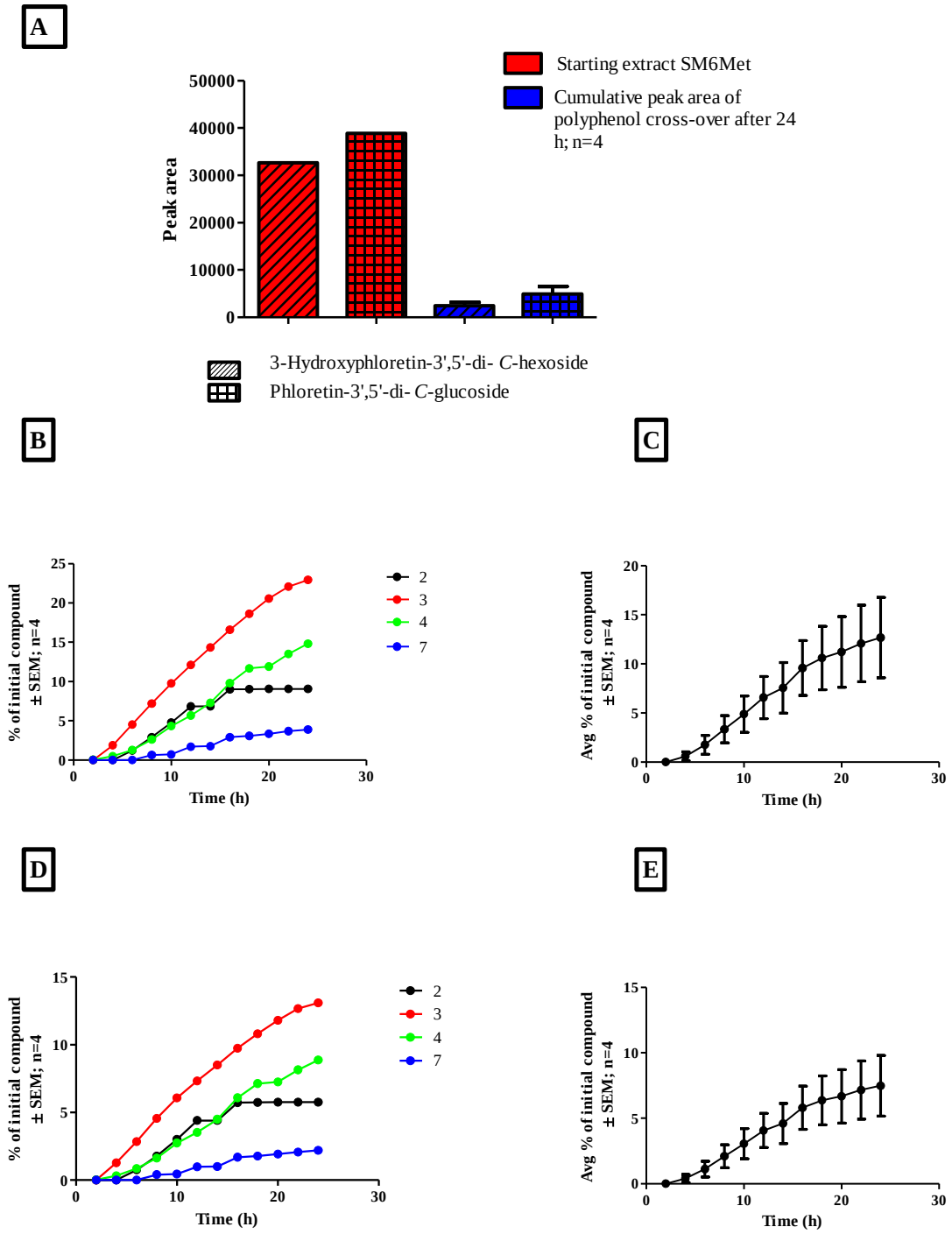

Figure 6 Diffusion analysis of the dihydrochalcones originating from SM6Met via porcine small intestine by means of comparing accumulated polyphenol small intestinal cross-over relative to its starting concentration. The permeability assay was performed at 37°C, over 24 h, using the flow-through diffusion system [16], with fractions collected at 2 h intervals. Fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) Peak area values displayed in red for the individual polyphenols as present in SM6Met, and cumulative peak areas displayed in blue, portraying the amount of the polyphenols that permeated the small intestine over 24 h.; Graphs B-E represent the cumulative peak area as a percentage of the polyphenol peak area in SM6Met at t = 0; B) individual replicate contribution to phloretin-3',5'-di-*C*-glucoside; C) average replicate contribution of phloretin-3',5'-di-*C*-glucoside; D) replicate contribution of 3-hydroxyphloretin-3',5'-di-*C*-hexoside; E) average replicate contribution of 3-hydroxyphloretin-3',5'-di-*C*-hexoside.

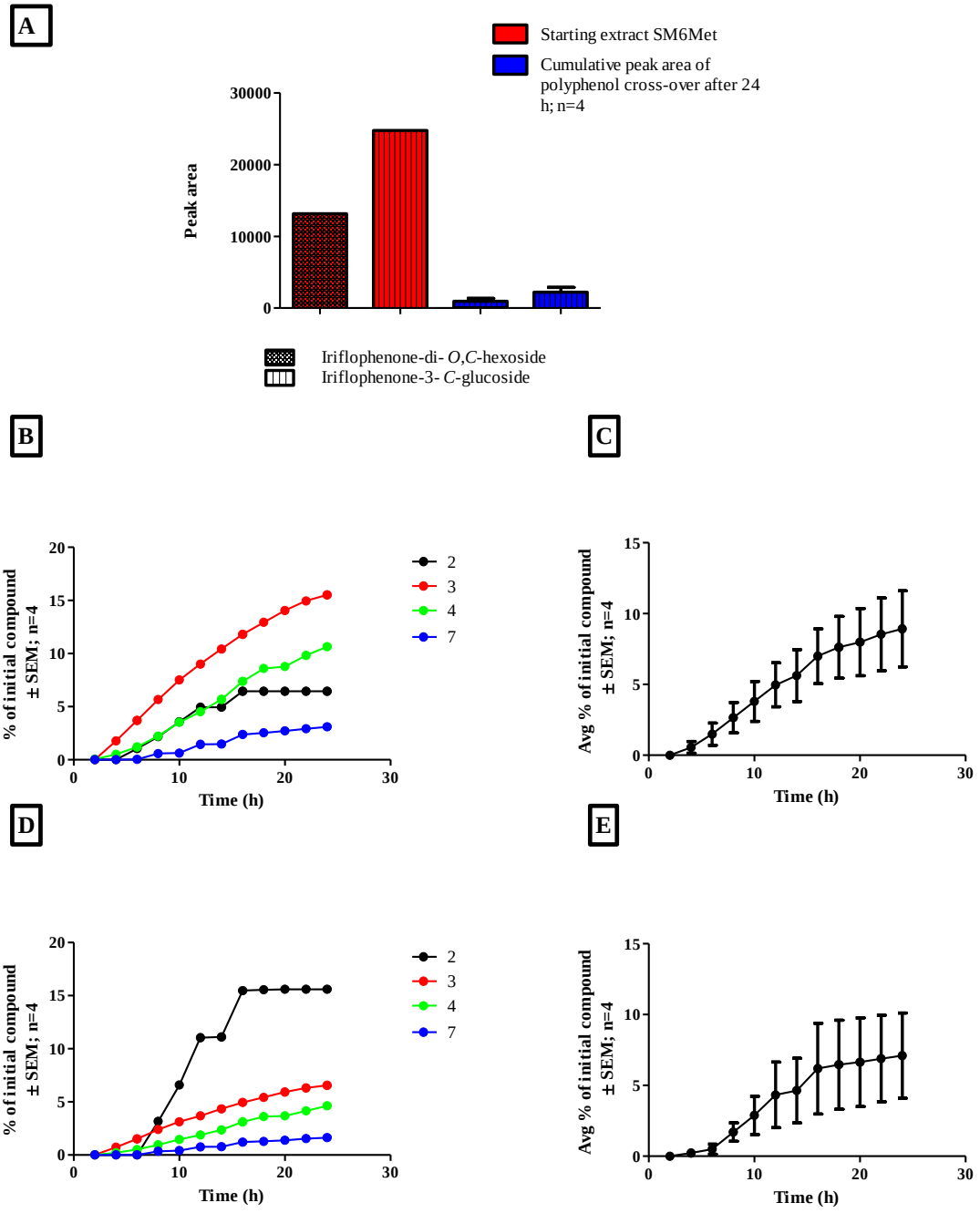

Figure 7 Diffusion analysis of the benzophenones originating from SM6Met via porcine small intestine by means of comparing accumulated polyphenol small intestinal cross-over relative to its starting concentration. The permeability assay was performed at 37°C, over 24 h, using the flow-through diffusion system [16], with fractions collected at 2 h intervals. Fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) Peak area values displayed in red for the individual polyphenols as present in SM6Met, and cumulative peak areas displayed in blue, portraying the amount of the polyphenols that permeated the small intestine over 24 h.; Graphs B-E represent the cumulative peak area as a percentage of the polyphenol peak area in SM6Met at t = 0; B) individual replicate contribution of iriflophenone-di-*O*,*C*-hexoside; C) average replicate contribution of iriflophenone-di-*O*,*C*-hexoside; D) individual replicate contribution of iriflophenone-3-*C*-glucoside; E) average replicate contribution of iriflophenone-3-*C*-glucoside.

In conclusion, even though large variability was observed between the individual replicates within the single experiment, it was possible to categorize the polyphenol compound classes present in

SM6Met in terms of their degree of diffusion across the porcine small intestinal mucosa. The dihydrochalcones displayed the highest average percentage of intestinal cross-over with 10.1 ± 3.2 %, followed by the benzophenones with 8.0 ± 2.9 %, the xanthones with 7.1 ± 2.4 % and the flavanones with 4.8 ± 1.7 %. The lowest percentage of diffusion was displayed by the flavones with 3.9 ± 1.6 %. The only polyphenols that were outliers within their respective groups were the flavanone, hesperidin (11.7 ± 3.9 %) and the flavone, scolymoside (11.5 ± 3.9 %).

4.3.3 Calculating select SM6Met polyphenol P_{app} values

The apparent permeability coefficients (P_{app}), which considers both the flux (J) and the initial concentration values (C_o in mg/mL), were calculated using the formula $P_{app}=J/C_o$ [22]. As we had previously (Chapter 3) determined the concentration of the polyphenols present in SM6Met using qHPLC (Table 1), we could quantify the intestinal cross-over concentration of these polyphenols. The concentrations of only some of the polyphenols in the positive control could be determined, and as such, the J and P_{app} values could only be calculated for these polyphenols.

Calculated P_{app} values, post-exclusion of replicates five and six, for the xanthones, mangiferin and isomangiferin, were 2.23 x 10^{-6} and 1.91 x10^{-6} cm/s, respectively; for the flavanones, eriocitrin and hesperidin, the values were 2.03 x 10^{-6} and 3.46 x 10^{-6} cm/s, respectively, whereas for the flavone, scolymoside, and the dihydrochalcones, phloretin-3',5'-di-*C*-glucoside and 3-hydroxyphloretin-3',5'-di-*C*-hexoside, the values were 3.39 x 10^{-6}, 3.7 x 10^{-6} and 2.2 x 10^{-6} cm/s, respectively (Table 1). The benzophenone, iriflophenone-3-*C*-glucoside displayed a P_{app} value of 2.64 (Table 1). Even though the trend of polyphenol P_{app} values stayed similar prior to (Table S2) and after exclusion (Table 1 & Table S2) of replicates five and six, an overall increase in values (Table 1) ranging from 38.22-44.26 % was observed when replicates five and six were excluded.

Table 1. Calculated flux and P_{app} values from quantified SM6Met polyphenols

Compound class	Polyphenol description	g/100g SM6Met	C_o	Q	Cumm peak area relative to positive control [a]	J/ Flux [b]	P_{app} [c]
Xanthone	Mangiferin	1.899	4.634	2.317	7.56	6.21	2.23
	Isomangiferin	0.645	1.574	0.787	6.47	1.80	1.91
Flavanone	Eriocitrin (eriodictyol-7-*O*-rutinoside)	0.846	2.064	1.032	6.86	2.51	2.03
	Hesperidin (hesperetin-7-*O*-rutinoside)	2.049	5.000	2.500	11.73	10.38	3.46
Flavone	Scolymoside (luteolin-7-*O*-rutinoside)	1.289	3.150	1.573	11.48	6.40	3.39
Dihydrochalcone	Phloretin-3',5'-di-*C*-glucoside	1.278	3.118	1.560	12.68	7.01	3.74
	3-Hydroxyphloretin-3',5'-di-*C*-hexoside	0.700	1.708	0.854	7.48	2.26	2.21
Benzophenone	Iriflophenone-3-*C*-glucoside	0.669	1.632	0.817	8.93	2.58	2.64

[a] refers to cumulated polyphenol peak area at 24 h relative to its peak area in the positive control
[b] refers to flux after 24 h with the values represented using the following units: x 10^2 ng.cm^{-2}.min^{-1}
[c] refers to P_{app} after 24 h with the values represented using the following units: x 10^{-6} cm.s^{-1}
Calculating J for mangiferin: [(0.01899g*0.122g)*1000000000)*7.56 /100)/1440 min]*1/0.196 cm^2=6.21 x 10^2 ng.cm^{-2}.min^{-1}
1.899g/100g was the concentration of mangiferin present in SM6Met
7.56 % was the cumulated peak area of mangiferin at 24 h relative to its peak area in the positive control
0.122 g was the amount of SM6Met loaded onto each replicate
1440 min = 24 h x 60 min
A = 0.196 cm^2
P_{app} = (6.21 x 10^2 ng.cm^{-2}.min^{-1}/60)/(0.01899g*0.244g*1000000000)= 2.23: x 10^{-6} cm.s^{-1}
Calculation of Q and Co was performed as described in section 4.2.6
C_o is represented in mg/mL
Q is represented as x 10^6 ng

There is currently no literature data available on the P_{app} values of these polyphenols regarding movement across intestinal mucosa. A general rule of thumb states that compounds with a P_{app} value larger than 2 x 10^{-6} cm/s are generally considered as being well absorbed in the human body [47]. In addition, other studies have indicated that compounds with P_{app} values higher than 1 x 10^{-6} cm/s display 100% absorption in the human body [48]. Interestingly, all of the polyphenols investigated, with the exception of isomangiferin (1.91 x 10^{-6} cm/s), displayed P_{app} values larger than 2 x 10^{-6} cm/s (Table 1), indicative of the fact that all of these polyphenols may be well absorbed in the human body.

4.3.4 Physicochemical properties of select SM6Met polyphenols

Comparing the physicochemical properties of SM6Met polyphenols (Table 2) with available literature allowed us to determine whether the investigated polyphenols would be fit for drug development as a possible phytoestrogenic nutraceutical. Specifically, we were interested in properties that would predict the extent of absorption in the human body. We were unable to obtain data for several of the polyphenols ((*S*)-eriodictyol-di-*C*-hexoside, (*R*)-eriodictyol-di-*C*-hexoside, naringenin-di-*C*-hexoside, luteolin-*O*-hexoside and iriflophenone-di-*O*,*C*-hexoside) investigated in the present absorption study. The OSIRIS program [25] was used to calculate polyphenol octanol-water partitioning coefficients (cLogP), solubility coefficients, 'drug-likeness' and overall drug-score, whereas the ACD/Labs software program [26] was used to calculate the molecular weight (MW), HBA, HBD, number of Lipinski's "Rule-of-5" violations (VI), rotating bonds (RB) and PSA. In addition to using the OSIRIS program, cLogP values were also calculated with the ACD/Labs and ChemAxon software programs [26] for comparison as values could differ between programs.

The cLogP value is accepted in drug design as a reliable measure of the hydrophilicity of a compound, where low hydrophilicity [49] corresponds to a high cLogP value. It is well known that compounds with a cLogP value greater than five are poorly absorbed in the human body [50]. Even

though large variation was observed between programs for the calculation of cLogP values, all SM6Met polyphenols displayed cLogP values lower than five, irrespective of software program used (Table 2). Values calculated with OSIRIS ranged from -3.12 for 3-hydroxyphloretin-3',5'-di-*C*-hexoside to 2.4 for luteolin. ACD/Labs values ranged from –2.57 for apigenin-6,8-di-*C*-glucoside to 2.4 for luteolin, and the ChemAxon values ranged from -2.81 for apigenin-6,8-di-*C*-glucoside to 2.4 for luteolin, thus indicating that all will be absorbed with relative ease in the human body when considering cLogP [50].

Solubility was another factor of concern as we know that higher solubility is correlated with increased P_{app} [9] and thus absorption. In addition, as indicated by the OSIRIS program, over 80 % of commercially available drugs have a calculated log S (solubility coefficient) value greater than -4. Again, this was evident for all of the compounds investigated (Table 2), with values ranging from -3.97 for isomangiferin to -1.04 for protocatechuic acid, again predicting good absorbance of all SM6Met polyphenols in the human body.

Compound drug-likeness is an indication of whether a compound has physical properties frequently present in commercial drugs, however this is irrespective of its lipophilicity [25]. About 80 % of commercially available drugs have a positive drug-likeness value, and it is thus suggested that a positive drug-likeness value is ideal [25]. The only polyphenols from SM6Met that exhibited negative drug-likeness values were the xanthones, mangiferin and isomangiferin, the flavanones eriodictyol-*O*-glucoside, the flavone, apigenin-6,8-di-*C*-glucoside, the dihydrochalcones, 3-hydroxyphloretin-3',5'-di-*C*-hexoside) and phloretin-3',5'-di-*C*-β-glucoside, the benzophenone, iriflophenone-3-*C*-glucoside and the phenolic acid, protocatechuic acid (Table 2). The majority of the polyphenols in SM6Met therefore exhibited the desired degree of druglikeness.

The overall drug-likeness score considers cLogP, logS, molecular weight and toxicity risks in one value that may be used to judge the overall potential of a compound to qualify for a drug [25]. Overall drug-likeness score values obtained for the SM6Met polyphenols were relatively low to

moderate with values ranging from 22 to 42 % for the xanthones, 44-59 % for the flavanones, 13-34 % for the flavones, 38-43 % for the phenolic acids, 32-34 % for the dihydrochalcones, and 21-22 % for the flavanols (Table 2). These low to moderate values may be attributed to the fact that some polyphenols did not exhibit drug-likeness characteristics as evidenced by a negative drug-likeness score. In addition, some polyphenols display moderate to severe toxicity risks which comprises of tumorigenic (luteolin, quercetin-*O*-rutinoside, and quercetin-*O*-neohesperidoside (moderate)), irritant (isomangiferin (severe)), mutagenic (protocatechuic acid (severe), *p*-coumaric acid (moderate), scolymoside (severe), luteolin (severe), quercetin-*O*-rutinoside (severe), chrysoeriol-*O*-rutinoside (severe), apigenin-6,8-di-*C*-glucoside (severe), quercetin-*O*-neohesperidoside (severe), chrysoeriol-*O*- neohesperidoside (severe), apigenin-O- neohesperidoside (severe)) and reproductive affecting risks (*p*-coumaric acid (severe), luteolin (moderate), quercetin-*O*-rutinoside (moderate), apigenin-6,8-di-*C*-glucoside (severe), quercetin-*O*-neohesperidoside (moderate), apigenin-*O*-rutinoside (severe), apigenin-*O*-neohesperidoside (severe)) (Table 3) [25].

The ACD/Labs program uses the number of Lipinski's "Rule-of-5" violations [50] as a measure of the absorption potential and drug-likeness of the compound of interest. Literature suggests that if a compound violates more than 1 of the rules it will display poor absorption in the human body [50]. For any of the "Rule-of-5" to be violated, the compound must display either a molecular weight greater than 500 g/mol, a cLogP value more than five, number of HBD more than 5, and number of HBA more than 10. In addition, if the PSA is greater than 140 $Å^2$ and the number of RB is more than ten, the compound is likely to display poor absorption, unless these compounds are actively transported [7,8]. In the case of the xanthones, mangiferin and isomangiferin, two of the "Rule-of-5" parameters are violated (Table 2), namely the number of HBA and HBD, indicating poor absorption. In addition, the PSA for both compounds is above 140$Å^2$. For the investigated flavanones, the number of parameter violations ranged from 2 for eriodictyol-*O*-glucoside to 3 for eriocitrin and hesperidin. The cut-off for the number of HBD and acceptors are exceeded in all cases, and in addition, the molecular weight cut-off is exceeded by eriocitrin and hesperidin.

Furthermore, poor absorption for all three compounds is indicated, based on their PSA exceeding 140 $Å^2$, and the number of RB exceeding 10 (Table 2). All of the flavones, with the exception of luteolin, display three parameter violations of the "Rule-of-5", i.e. molecular weight, and number of HBD and HBA. Their PSA and number of RB also exceed the recommended value for good absorption. Luteolin is the only flavone that passes the "Rule-of-5" and stays within the limits for PSA and number of RB.

Data available for the flavonol quercetin-*O*-rutinoside/neohesperidoside and the phenolic acid, protocatechuic acid, indicate poor absorption as both violated 3 Lipinski "Rule-of-5" parameters. Additionally the flavonol, but not the phenolic acid, exceeds the limits for PSA and number of RB (Table 2).

Table 3. Toxicity risks of polyphenols as indicated by the OSIRIS program [25]

Polyphenol description	Toxicity Risks			
	Irritant	Mutagenic	Tumorigenic	Reproductive affecting
Luteolin		S	M	M
Quercetin-*O*-rutinoside		S	M	M
Queretin-*O*-neohesperidoside		S	M	M
Isomangiferin	S			
Protocatechuic acid		S		
p-Coumaric acid		M		S
Scolymoside (luteolin-7-*O*-rutinoside)		S		
Chrysoeriol-*O*-rutinoside		S		
Chrysoeriol-*O*-neohesperidoside		S		
Apigenin-6,8 di-*C*-glucoside		S		S
Apigenin-*O*-neohesperidoside		S		S
Apigenin-*O*-rutinoside				S

M refers to moderate
S refers to severe
Compounds not allocated a toxicity risk rating as for certain compounds above did not display toxicity risks.
As there is uncertainty about whether the sugar moeity for apigenin-O-rutinoside/neohesperidoside and chrysoeriol-O-rutinoside/neohesperidoside compounds were investigated all the aglycones containing either sugar moeities were investigated separately.

Table 2. Physicochemical properties of select SM6Met polyphenols

Compound description	OSIRIS				ACD/Labs software							CH[a]
	CLP[b]	SOL[c]	DL[d]	DS[e]	MW[f]	HBA[g]	HBD[h]	CLP	VI[i]	RB[j]	PSA[k]	CLP
A: Mangiferin	0.11	-2.57	-4.28	0.42	422.3	11	8	0.13±1.29	2	10	197.37	-0.36
Isomangiferin	1.22	-3.97	-4.37	0.22	422.3	11	8	0.223	2	10	197.37	-0.36
B: Eriodictyol-*O*-glucoside	0.09	-2.23	-2.21	0.44	450.4	11	7	0.22±0.83	2	11	186.37	0.26
Eriocitrin (eriodictyol-7-*O*-rutinoside)	-0.8	-2.44	3.26	0.59	596.5	15	10	2.089	3	16	256.29	-0.64
Naringenin-*O*-dihexoside												
Hesperidin (hesperetin-7-*O*-rutinoside)	0.02	-3.21	3.65	0.55	610.6	15	8	1.78±0.72	3	15	234.29	-0.31
C: Apigenin-6,8-di-*C*-glucoside (vicenin-2)	-2.57	-1.68	-1.26	0.13	594.5	15	11	-0.10±0.94	3	16	267.29	-2.81
Scolymoside (luteolin-7-*O*-rutinoside)	-0.61	-2.65	2.41	0.34	594.5	15	9	2.02±0.85	3	15	245.29	-0.94
Apigenin-7-*O*-rutinoside[#]	-0.31	-2.95	3.18	0.21	578.5	14	8	1.055	3	14	225.06	-0.29
Apigenin-*O*-neohesperidoside[#]	-0.31	-2.95	1.94	0.2	578.5	14	8	1.72±0.82	3	14	225.06	-0.29
Chrysoeriol-*O*-rutinoside[#]	-0.42	-2.97	1.64	0.31	NA	NA	NA	NA	NA	NA	NA	NA
Chrysoeriol-*O*-neohesperidoside[#]	-0.42	-2.97	2.21	0.33	NA	NA	NA	NA	NA	NA	NA	NA
Luteolin	2.4	-2.56	1.9	0.32	286.2	6	4	2.40±0.65	0	5	107.22	2.40
D: Iriflophenone-3-*C*-glucoside	-0.58	-1.95	-1.7	0.49	NA	NA	NA	NA	NA	NA	NA	NA
E: 3-Hydroxyphloretin-3',5'-di-*C*-hexoside	-3.12	-1.05	-1.89	0.34	NA	NA	NA	NA	NA	NA	NA	NA
Phloretin-3', 5'-di-*C*-glucoside	-2.82	-1.35	-2.27	0.32	NA	NA	NA	NA	NA	NA	NA	NA
F: Quercetin-*O*-rutinoside[#]	-0.95	-2.4	3.31	0.22	610.5	16	10	1.76±1.48	3	16	265.52	-0.87
Quercetin-*O*-neohesperidoside[#]	-0.95	-2.4	2.06	0.21	610.5	16	10	-0.356	3	16	155.52	-0.87
G: *p*-Coumaric acid	1.33	-1.7	0.58	0.38	NA	NA	NA	NA	NA	NA	NA	NA
Protocatechuic acid	0.9	-1.04	-0.12	0.43	154.1	4	3	1.16±0.24	0	3	77.76	1.02

A refers to the xanthones compound class
B refers to the flavanones compound class
C refers to the flavones compound class
D refers to the benzophenone compound class
E refers to the dihydrochalcone compound class
F refers to the flavanol compound class

G refers to the polyphenolic acid compound class
[a] CH refers to the ChemAxon open source software program used
[b] CLP refers to cLogP
[c] SOL refers to the solubility coefficient in percentage
[d] DL refers to the drug-likeness score
[e] DS refers to the overall drug score
[f] MW refers to molecular weight in g/mol^{-1}
[g] HBA refers to the number of hydrogen bond acceptors
[h] HBD refers to the number of hydrogen bond donors
[i] VI refers to the number of Lipinski rule violations
[j] RB refers to the number of rotating bonds
[k] PSA refers the polar surface area in $Å^2$
[#] refers to the uncertainty of which sugar moiety belongs to the aglycone
NA refers to the absence of polyphenol information from the source program

In conclusion, the OSIRIS program indicated that all of the investigated polyphenols would display relatively high permeability, as indicated by cLogP values < 5, and also high solubility (directly proportional to high permeability), as indicated by solubility coefficient values > -4, accompanied by the fact that most of the polyphenols displayed a positive druglikeness value. However, the toxicity risks of these compounds as well as the negative druglikeness of some polyphenols resulted in a lower overall druglikeness score for most polyphenols. Only two compounds, namely eriocitrin and hesperidin displayed an overall drug score above 50%. In contrast the ACD/labs program indicated that all of the polyphenols investigated, with the exception of luteolin, would display poor absorption in the human body due to the fact that these compounds exceeded the allowed Lipinski "Rule-of-5" violation limit of 1.

4.4 Discussion

As phytoestrogenic nutraceuticals are seen as a safer alternative to traditional HRT for women in menopause [51-57] this has become a topic of interest in recent years. Even though *Cyclopia* species have shown potential for the development of a phytoestrogenic nutraceutical [10,11], a more recent study identified a specific *Cyclopia subternata* sequential methanol extract, SM6Met, as displaying the highest potency of tested *Cyclopia* extracts in terms of phytoestrogenic properties [12]. In addition, SM6Met displayed the positive estrogenic attributes of ERα antagonism, ERβ agonism and antagonism of E_2-induced breast cancer cell proliferation [13]. In the current study we evaluated the permeability of porcine small and large intestine for SM6Met polyphenols using the flow-through diffusion assay. This would assist in the search for polyphenols that may be absorbed through the human intestinal lining post oral intake and would be crucial for the development of a future oral phytoestrogenic nutraceutical containing select polyphenols originating from the SM6Met extract.

The most common *in vitro* assay evaluating intestinal permeability is performed using Caco-2 cells [58-61], which are adenocarcinoma cells derived from the colon, although some *in situ* perfusion

studies have been performed using rat intestines [62,63] and studies using oral administration in rats, pigs and rabbits [14,15,17,18,29,30,38-40,64]. We, however, made use of the flow-through diffusion assay, which has also been used for several absorption studies [23,65,66], albeit not for polyphenolic compounds such as investigated in the present study. Thus we for the first time evaluated the permeability of these two porcine tissue types a using the flow-through diffusion assay for polyphenols originating from SM6Met at concentrations present in the extract. The pig is regarded as an excellent model for human absorption due to its physiology being more similar to those of humans than any other non-primate mammalian models [67].

Even though previous studies have been performed on the absorption of some of the xanthones, flavanones, flavones, dihydrochalcones and benzophenones present in SM6Met, these studies had a more holistic approach as they considered not only intestinal absorption but also metabolism of these polyphenols in a more complex mice, rat or pig *in vivo* system [14,15,17,18,29,30,36,38-40,64]. In most of these studies the polyphenols that were orally administered were not detected in the blood plasma or urine, however, metabolites or conjugates of these polyphenols were present, which is indicative of absorption [14,15,17,18]. In some cases, mostly studies in rats, intact molecules not affected by hepatic metabolism or intestinal degradation were found in the plasma [17,18,38-40]. In our study we showed that even though we did not detect absorption of polyphenols from porcine large intestine, we detected all of the SM6Met polyphenols, with the exception of luteolin and quercitin rutinoside/neohesperidoside, in fractions collected from the small porcine intestine experiment. We postulate that the reason permeation of polyphenols through the large intestine was not observed may be due to the fact that in an *in vivo* system the large intestine polyphenols are hydrolysed by intestinal microflora yielding small polyphenolic acids followed by circulatory absorption, and as the microflora is not present in the *ex vivo* flow-through diffusion assay, the unmodified polyphenols might not physically be able to permeate the large intestinal barrier. For the small intestine experiment, results obtained suggest that during the 24 h period an average of 7.1 % of the xanthones found in the SM6Met extract moved across the porcine

small intestine, while for the flavanones and flavones this was 4.8 % and 3.1 %, respectively, and for the dihydrochalcones and benzophenones it was 10.1 % and 8.0 %, respectively. As all of the above mentioned compounds are glycosides, with the exception of luteolin, it is important to note that in an *in vivo* system *C*-glycosides are less frequently hydrolysed by intestinal glucosidases than *O*-glycosides [20], whereas a compound with a rutinoside moiety is commonly not hydrolysed in the small intestine, but by the colonic microflora, resulting in less optimal absorption of the aglycone as a whole due to partial polyphenol degradation [68]. In contrast, results obtained in the *ex vivo* flow-through diffusion assay indicated that compound classes with the highest permeation percentages were the classes with compounds containing *C*-glycosides, the dihydrochalcones, benzophenones and the xanthones. Furthermore, compounds with a rutinoside moeity did not generally exhibit lower permeation percentages.

An important factor that has not been accounted for in the current study is the role of UDP-glucuronosyltransferases (UGTs) in the absorption of polyphenols. These enzymes are mainly expressed in the liver and intestine, and they are responsible for compound glucoronidation [69,70]. It has been shown that glucoronidation of phenolic compounds, such as the polyphenols investigated in the current study, results not only in impaired absorption of these compounds [69,71], but also in some instances in the lowering of their biological activities [72]. Furthermore, UGTs has been shown to catalyze the glucuronidation of phenolic compounds in a species-, gender-, organ-, and isoform-dependent manner [69]. The current absorption study did not incorporate detection parameters for glucoronidated polyphenol conjugates, and as such we did not investigate the effect of intestinal UDP-glucuronosyltransferases on the permeation of the relevant polyphenols.

Most *in vitro* intestinal permeation studies previously performed on some of the polyphenols present in SM6Met used Caco-2 cells [41-43] as they resemble human small intestinal cells morphologically and functionally. Some disadvantages are, however, coupled with the use of this model which might alter intestinal performance. These disadvantages include the lack of cell

heterogeneity, the lack of the mucus layer of the intestinal wall and the absence of expression of some intestinal enzymes and transporters [73,74]. Thus the use of excised porcine small intestine in the current *in vitro* model should overcome these limitations and enabled us to obtain P_{app} values for all major polyphenols and polyphenols of interest in the SM6Met extract. The P_{app} values we obtained ranged from 1.61-3.74 x 10^{-6} $cm.s^{-1}$correlating with good absorption in the human body [47]. In contrast to the *in vitro* absorption study, evaluation of the physicochemical properties of the polyphenols in SM6Met using the ACD/Labs computational program predicted more than two Lipinski "Rule-of-5" violations for all polyphenols investigated, except for luteolin, suggesting that these polyphenols would be poorly absorbed in the human body, whereas luteolin may be absorbed well. In addition, even though the OSIRIS and ChemAxon programs indicated good permeability (cLogP values < 5) coupled to high polyphenol solubility (log S > -4), the OSIRIS program predicted, considering most commercially available drugs as reference, a low to moderate overall drug score for all the investigated polyphenols. As predicted polyphenol physicochemical properties vary between programs, it is evident that one should enrol as many different programs as possible and compare the results obtained to physiologically more relevant models for a more accurate prediction of the absorption of a compound in the human body.

Future work should investigate less complex extracts of SM6Met, as discussed in the previous chapter, which could include CCC-obtained F1, F2 and F3 where the investigated polyphenols are already linked to a specific estrogenic activity, as these permeation studies would serve as a preliminary qualification step for these compounds to form part of an intelligent mixture as an oral nutraceutical. *In vivo* studies after oral gavage of these less complex mixtures should also be performed for correlation of these results with a preliminary permeation study, as an *in vivo* study would not only investigate permeability but also metabolism of the polyphenols of interest. A recent *in vivo* study, completed with SM6Met (Appendix 1), indicated that SM6Met polyphenols were absorbed. Oral gavage administration of the extract resulted in a delay of rat vaginal opening, and

also a decrease in uterus size in rats treated with the extract and E_2, compared to uterus size of rats treated with E_2 alone [13]

In conclusion, even though not seen for the porcine large intestine, all of the polyphenols present in quantifiable concentrations in the SM6Met extract permeated through the porcine small intestine, demonstrating that no structural or physicochemical deterrent exists for these polyphenols to do so. This paves the way for future studies, where a more holistic approach could be taken by evaluating the absorption of these compounds in an *in vivo* animal model similar to that of the human system. As mentioned in the previous chapter, it seems unlikely that one compound could explain all of the positive estrogenic attributes of SM6Met. Furthermore, the concept of formulations consisting of intelligent mixtures of natural products to treat diseases has become a popular focus of recent studies [75-80]. Thus the formulation of an intelligent mixture from SM6Met is probably needed, where after this mixture would need to be evaluated for intestinal absorption.

4.5 Bibliography

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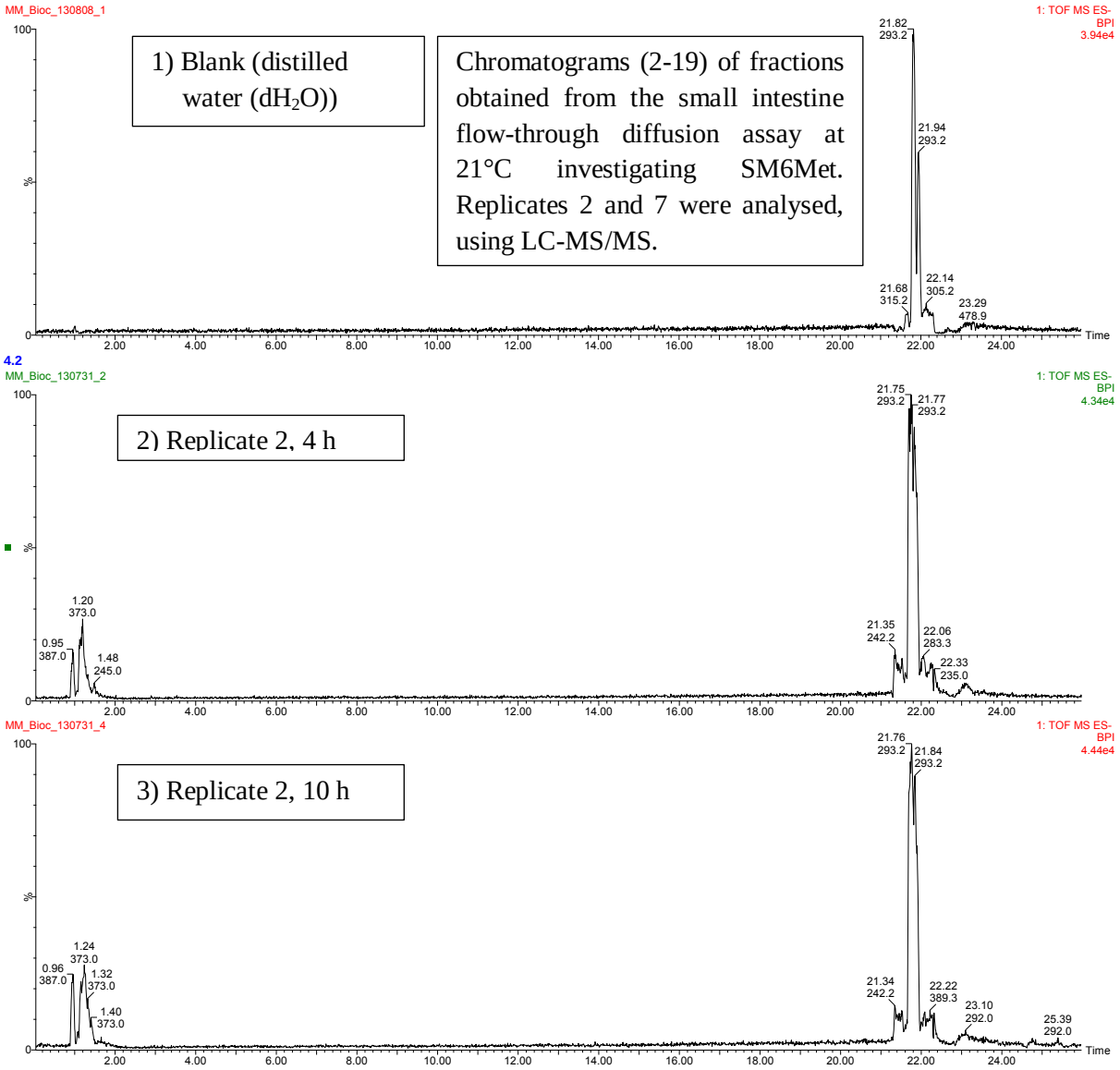
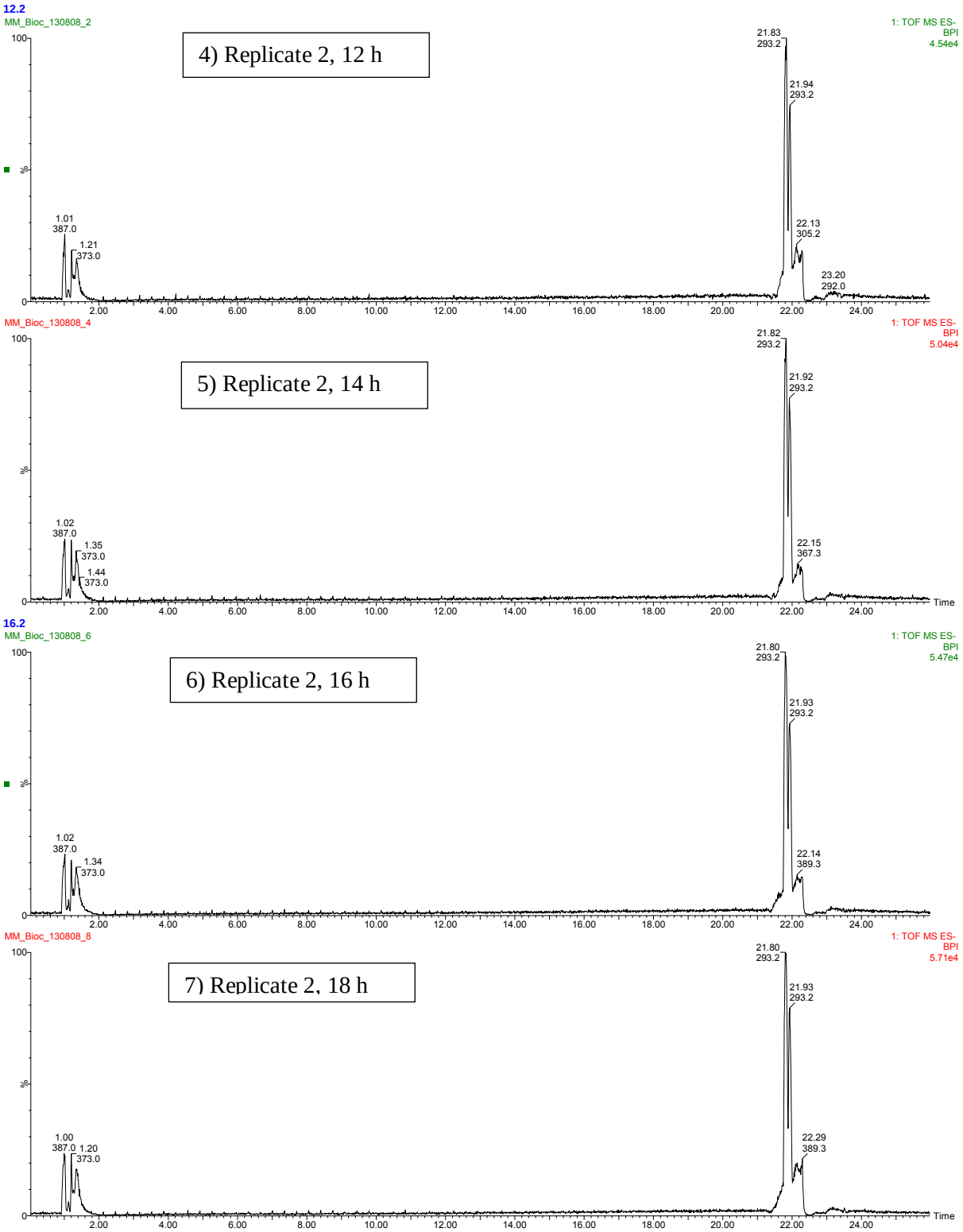
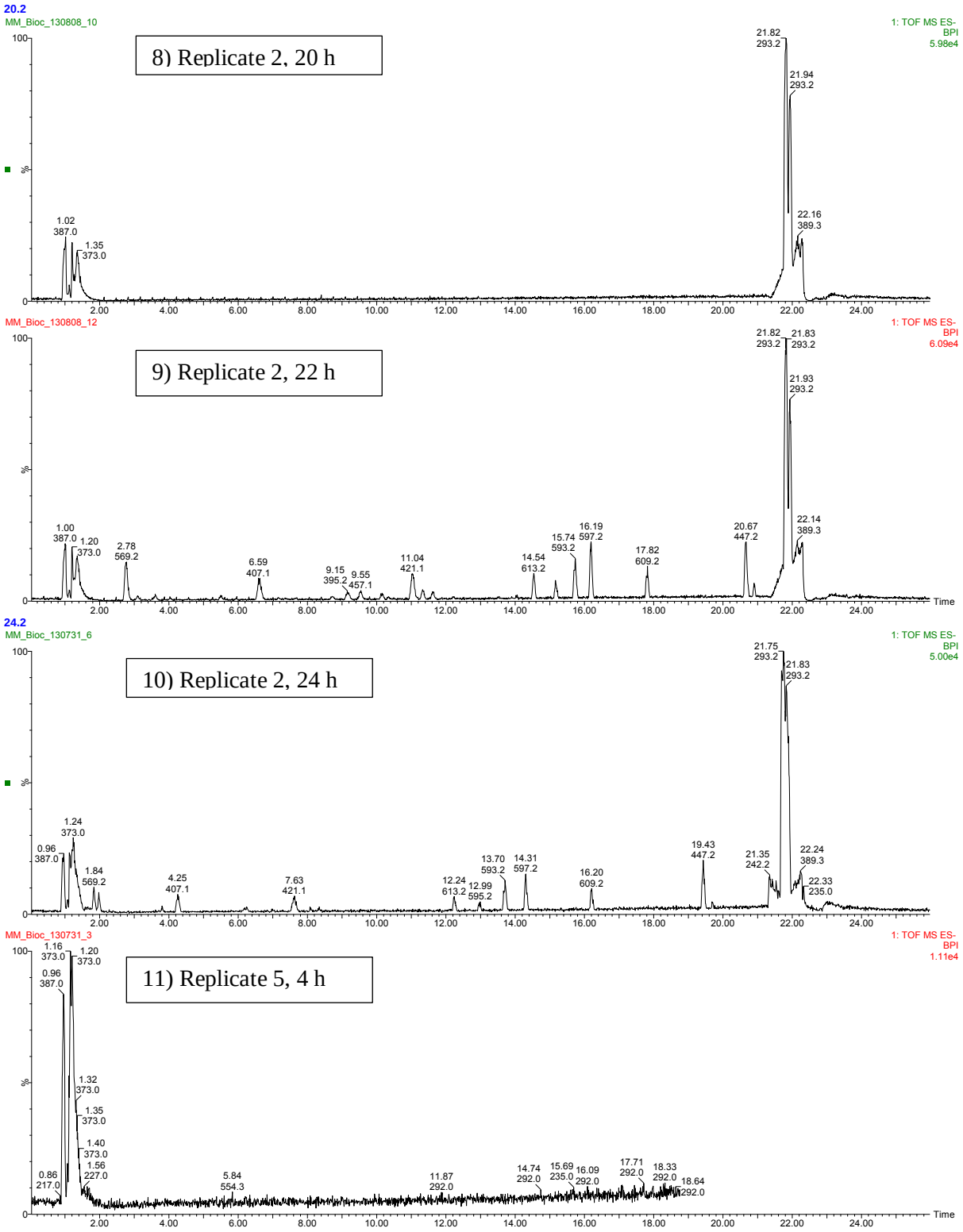
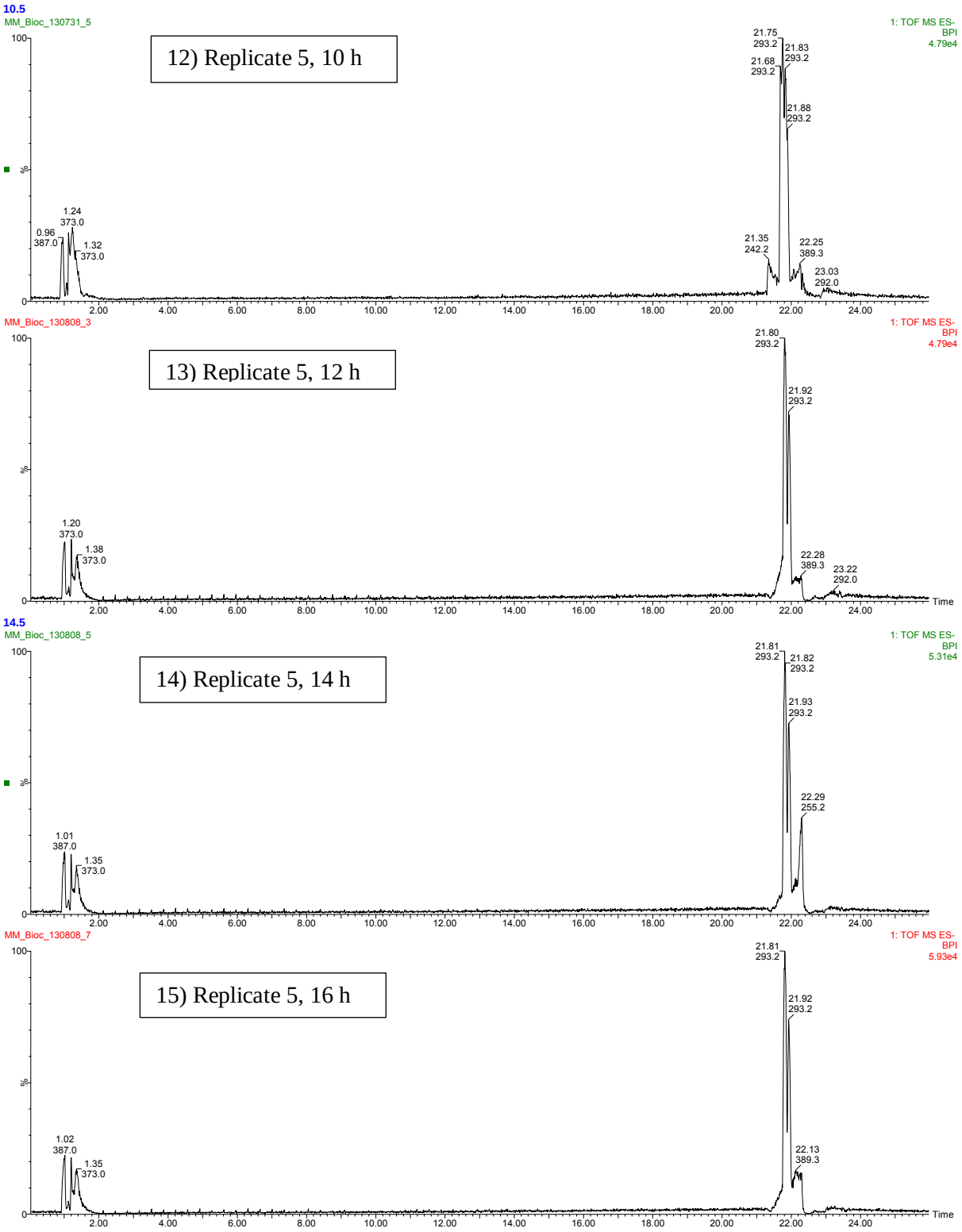
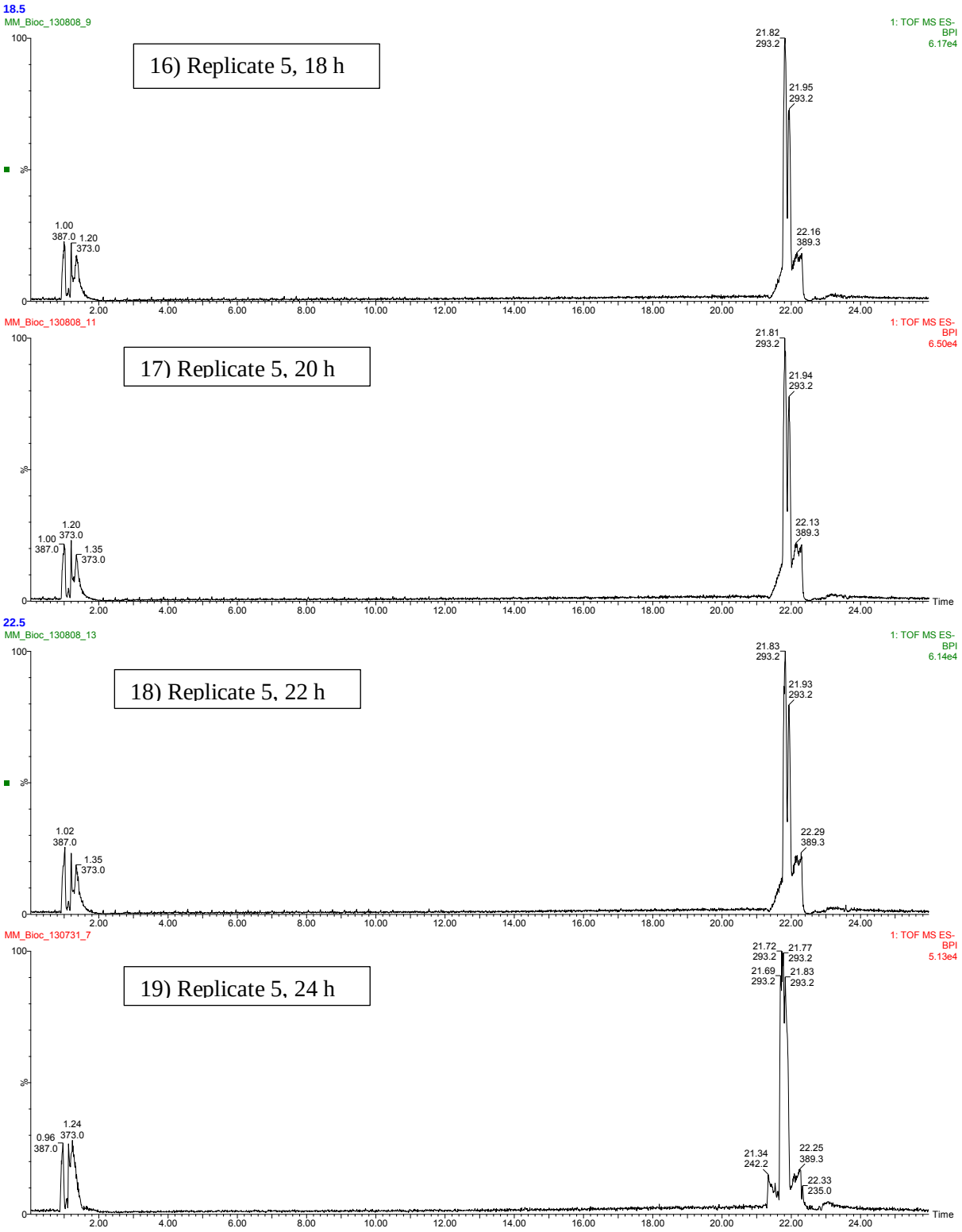
4.6 Supplementary Data

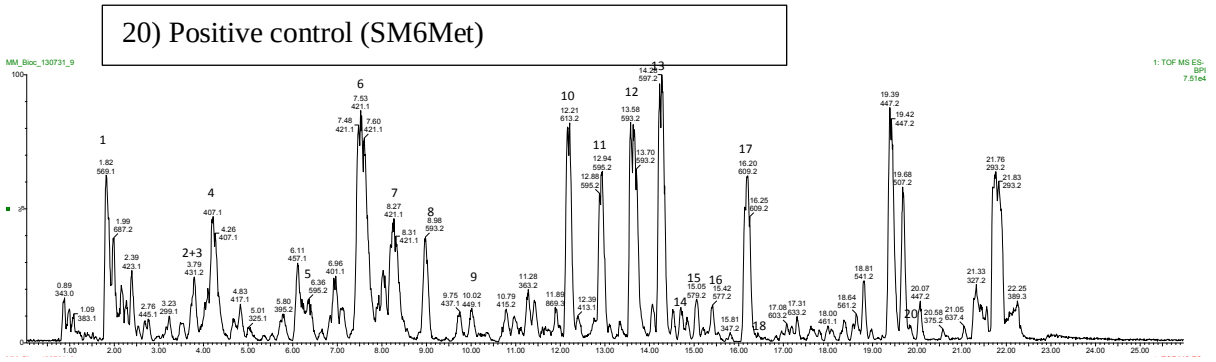












Supplementary Figure 1. Chromatograms obtained from 21°C small intestine flow-through diffusion assay fractions LC-MS/MS analysis of fractions collected every two hours for 24 h for replicates two and five. Chromatograms 2-19 represent replicates two and five at the 12, 2 h time intervals for 24 h, represented as % base peak intensity over time. Chromatogram 1 represented the blank (distilled H_2O) and chromatogram 20 the positive control (244 mg/mL, 10 x diluted) containing 9.1 mg/mL ascorbic acid.

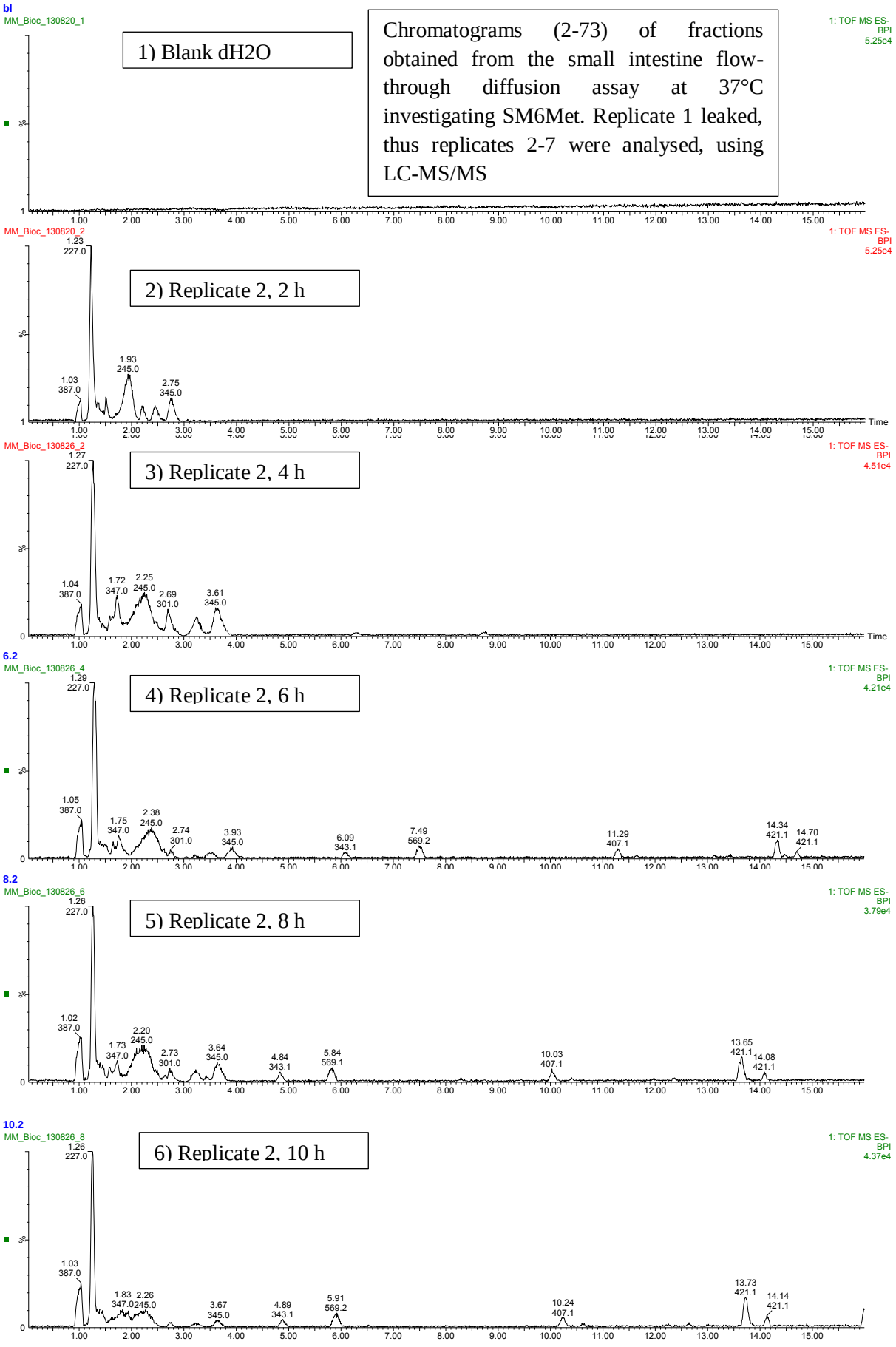
Supplementary Table 1. Polyphenols present in a postive control sample for the flow-through diffusion assay performed investigating porcine small intestine permeability for SM6Met polyphenols at 21°C

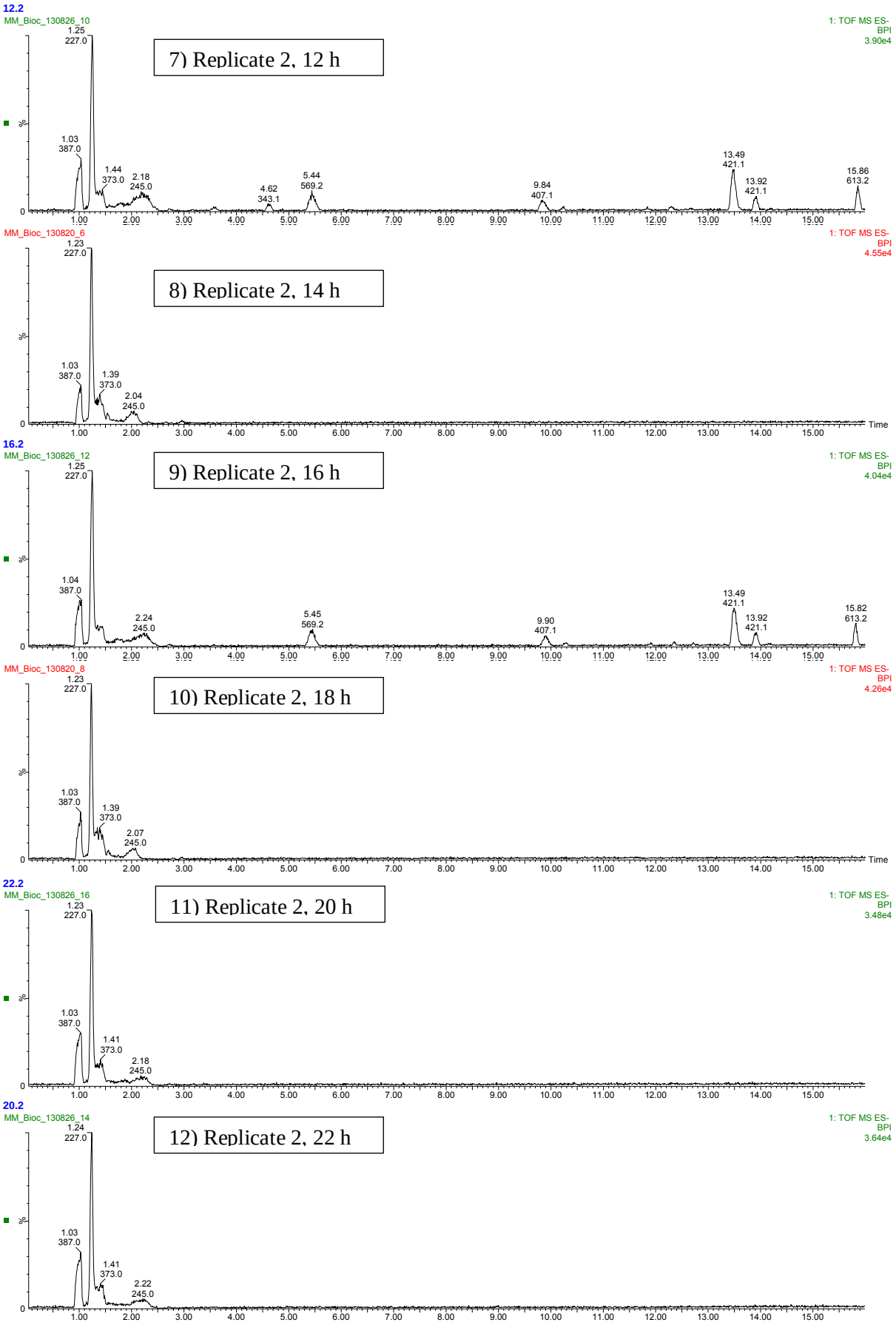
Number	Retention time	$[M-H]^-$	Compound
1	1.8	569	Iriflophenone-di-*O*, *C*-hexoside
2	3.5	611	(*S*)-Eriodictyol-di-*C*-hexoside
3	3.6	611	(*R*)-Eriodictyol-di-*C*-hexoside
4	4.2	407	Iriflophenone-3-*C*-glucoside
5	6.4	595	Naringenin-di-*C*-hexoside
6	7.5	421	Mangiferin
7	7.6	421	Isomangiferin
8	9.0	593	Apigenin-6,8-di-*C*-glucoside (vicenin-2)
9	10.0	449	Eriodictyol-*O*-glucoside
10	12.2	613	3-Hydroxyphloretin-3',5'-di-*C*-hexoside
11	12.9	595	Eriocitrin (eriodictyol-7-*O*-rutinoside)
12	13.6	593	Scolymoside (luteolin-7-*O*-rutinoside)
13	14.3	597	Phloretin-3',5'-di-*C*-glucoside
14	14.6	447	Luteolin-*O*-hexoside
15	15.1	579	Naringenin-*O*-dihexoside
16	15.4	577	Apigenin-*O*-rutinoside/neohesperidoside
17	16.2	609	Hesperidin (hesperetin-7-*O*-rutinoside)
18	16.4	607	Chrysoeriol-*O*-rutinoside /neohesperidoside
19	NA	609	Quercetin-*O*-rutinoside/neohesperidoside
20	19.8	285	Luteolin

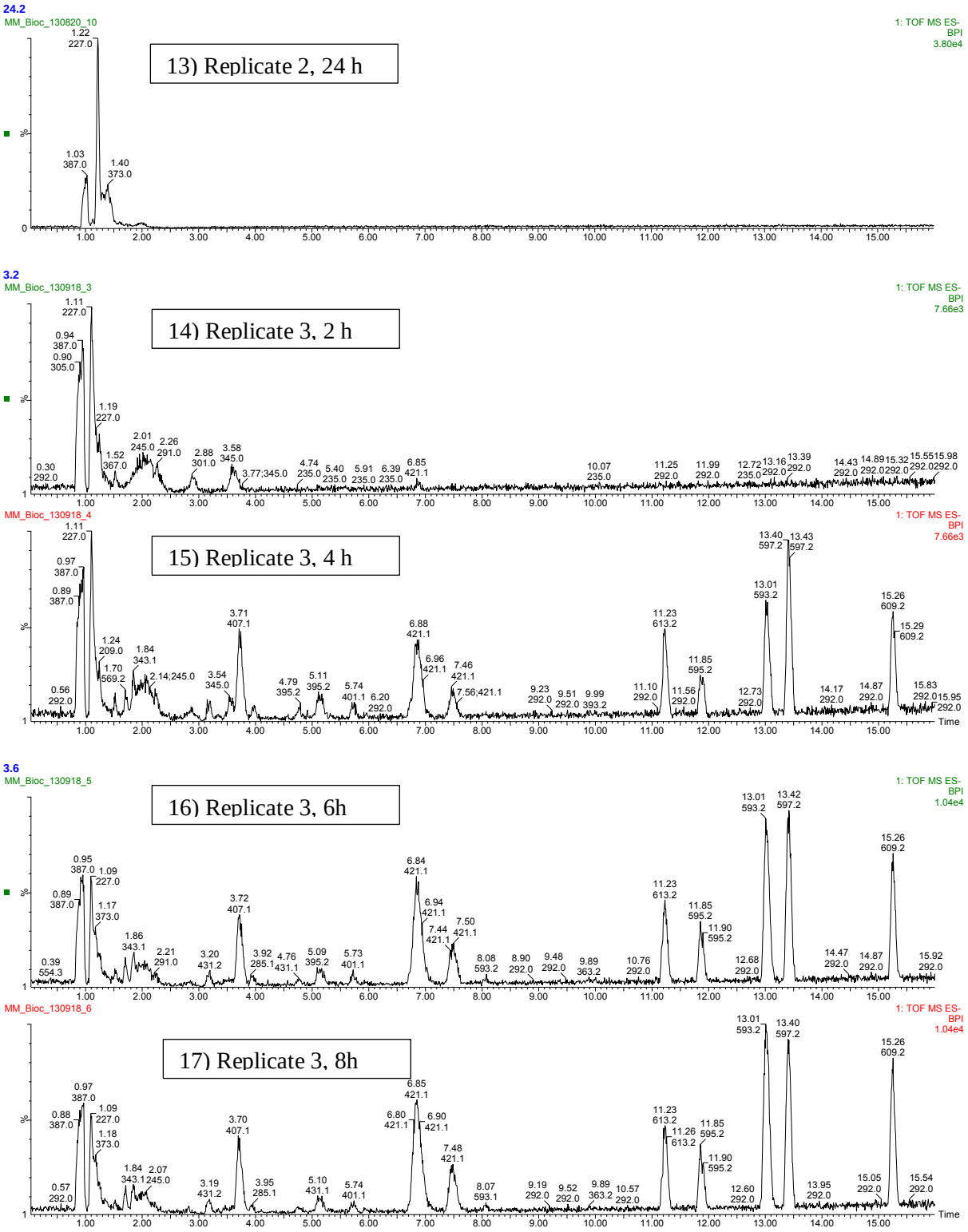
NA refers to the fact that the compound could not be detected in this SM6Met positive control

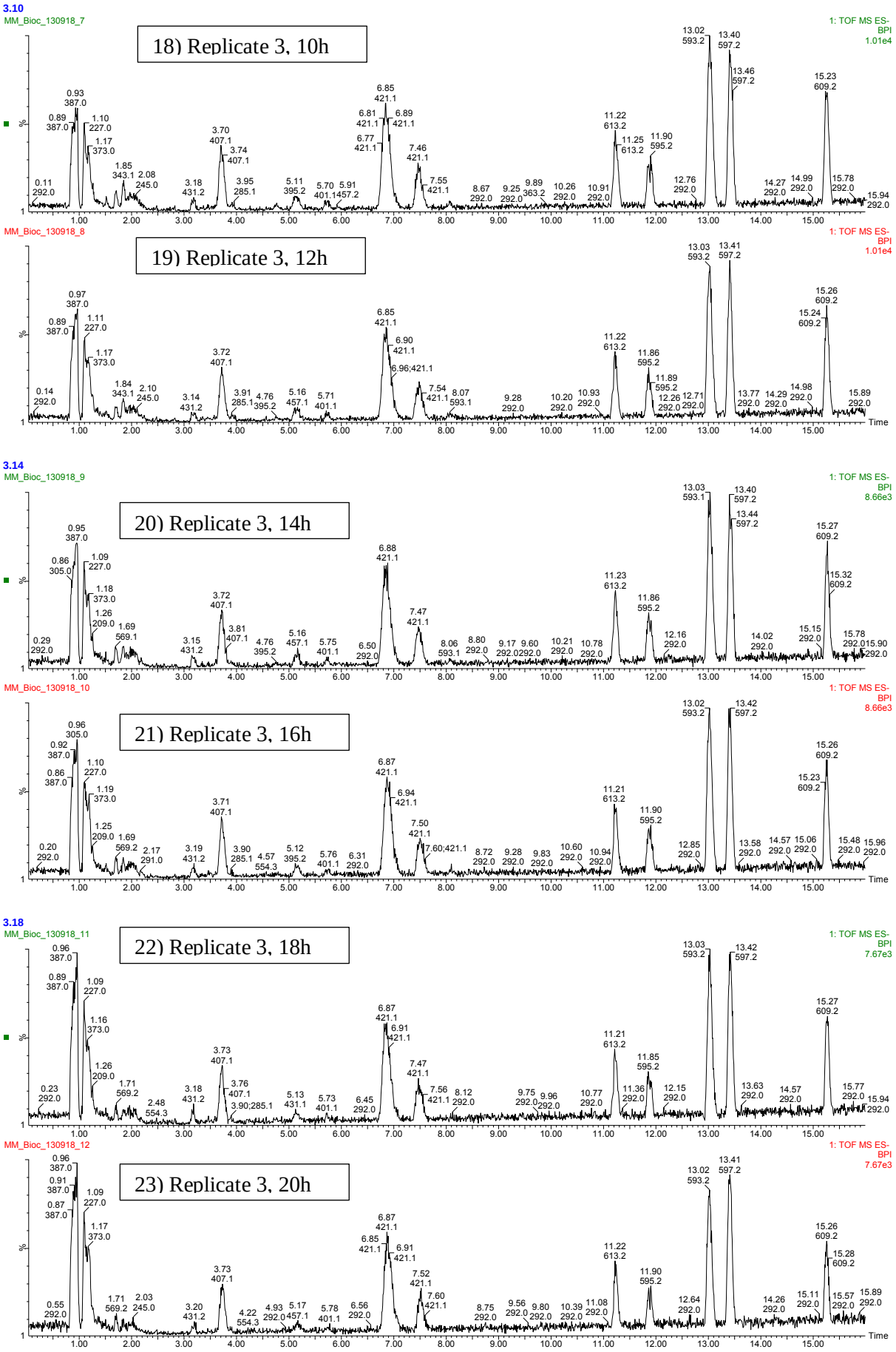
$[M-H]^-$ refers to compound molecular ion under the negative ionisation LC-MS/MS

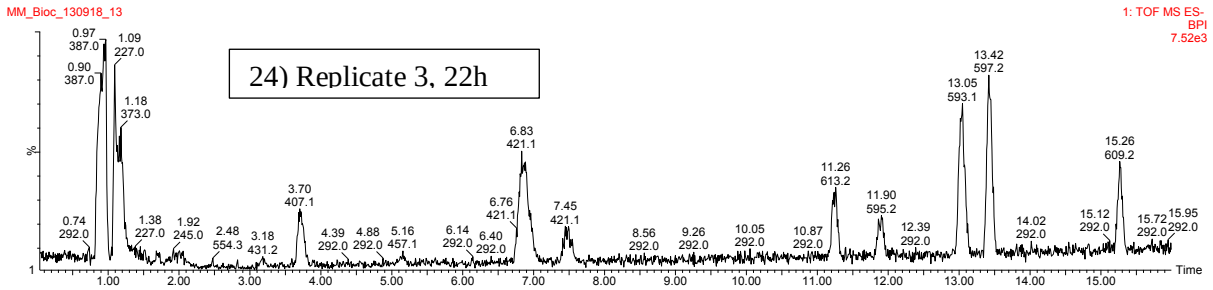
Chromatograms (2-73) of fractions obtained from the small intestine flow-through diffusion assay at 37°C investigating SM6Met. Replicate 1 leaked, thus replicates 2-7 were analysed, using LC-MS/MS

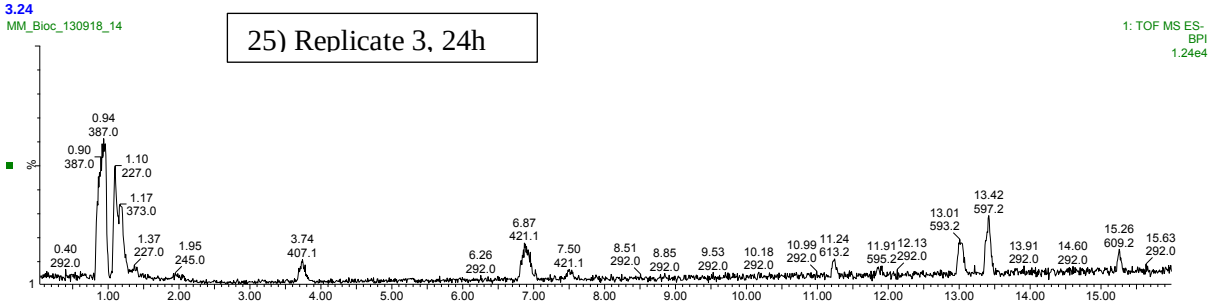


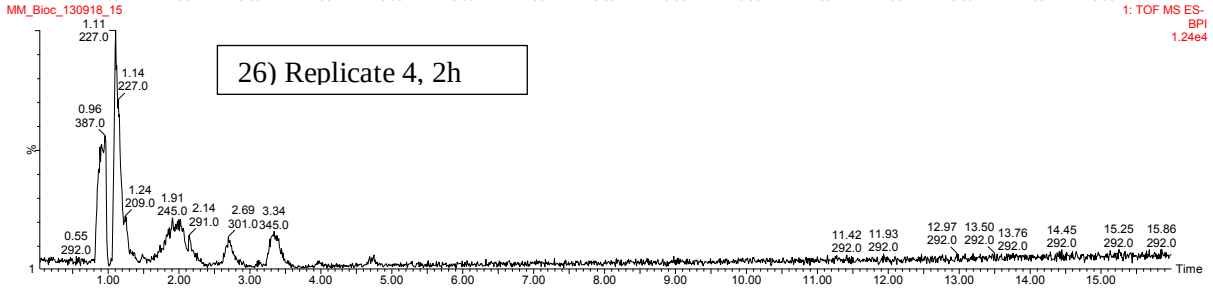


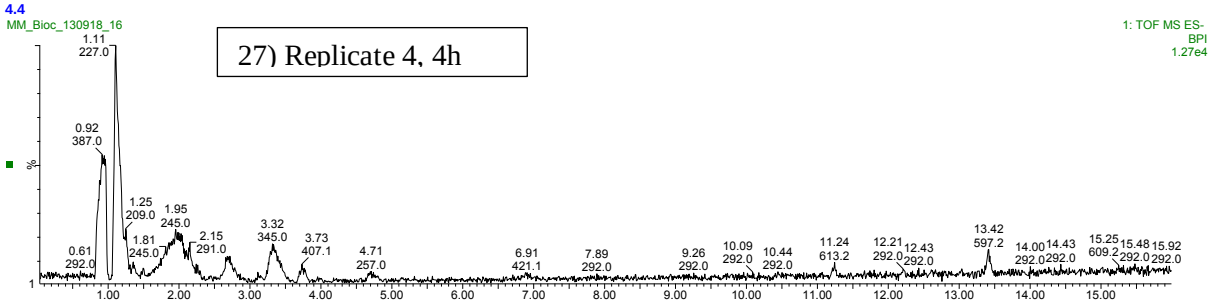


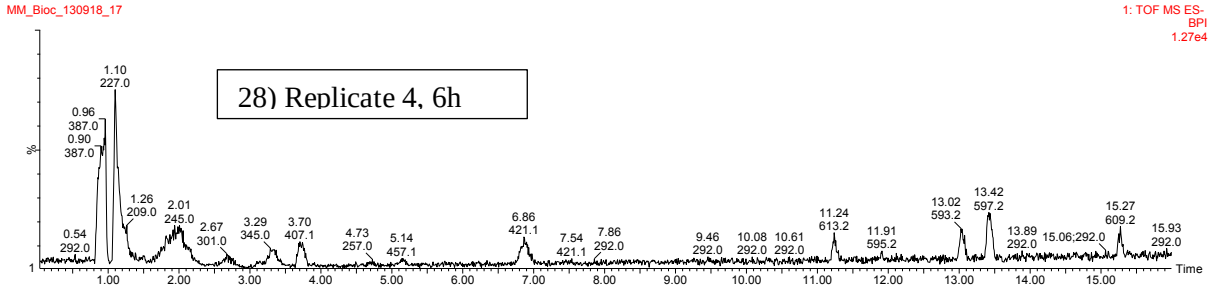


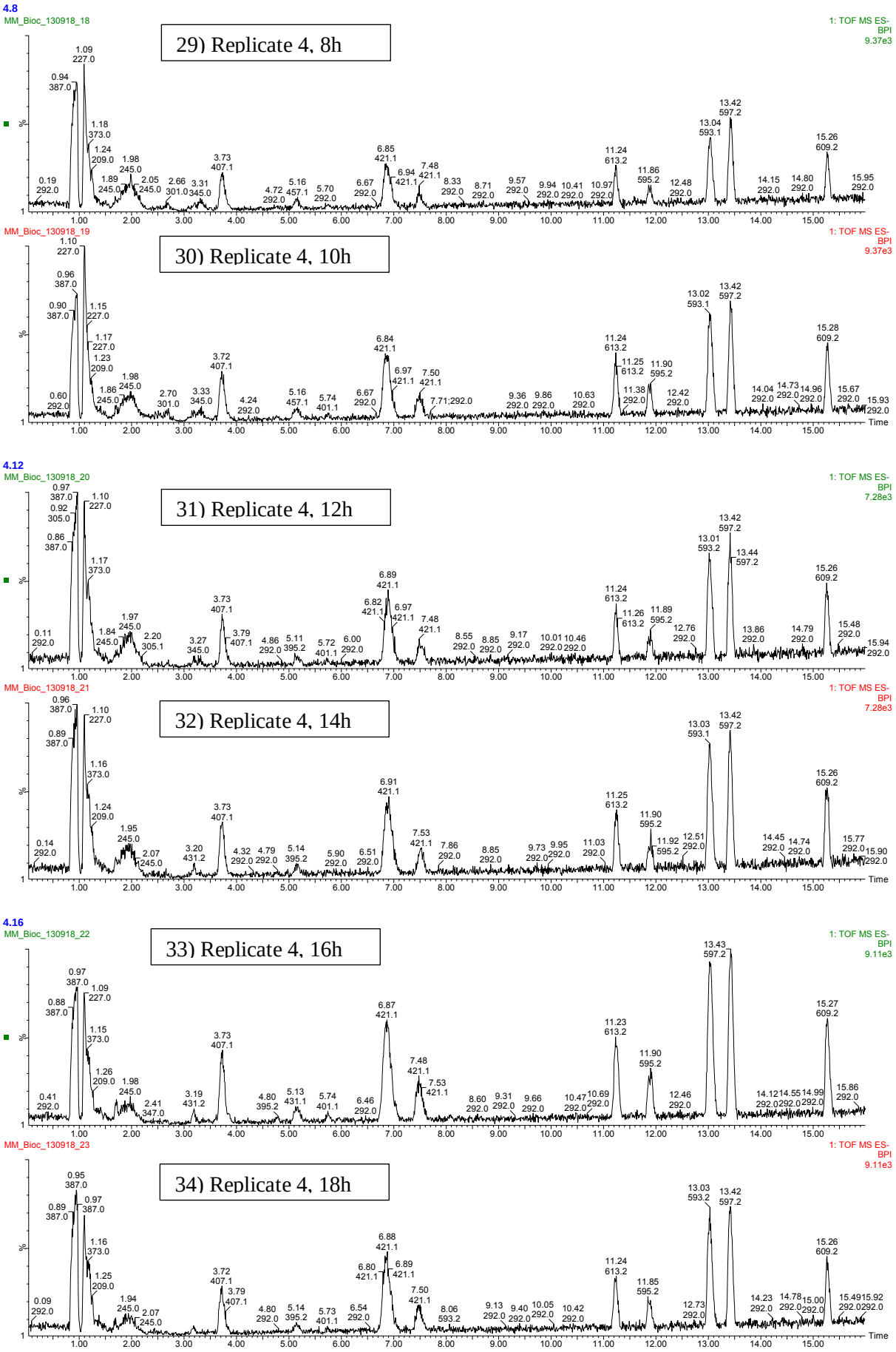


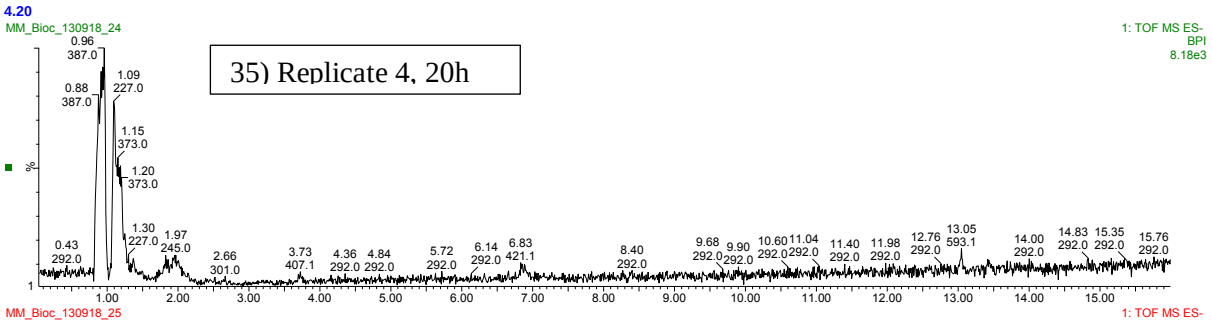


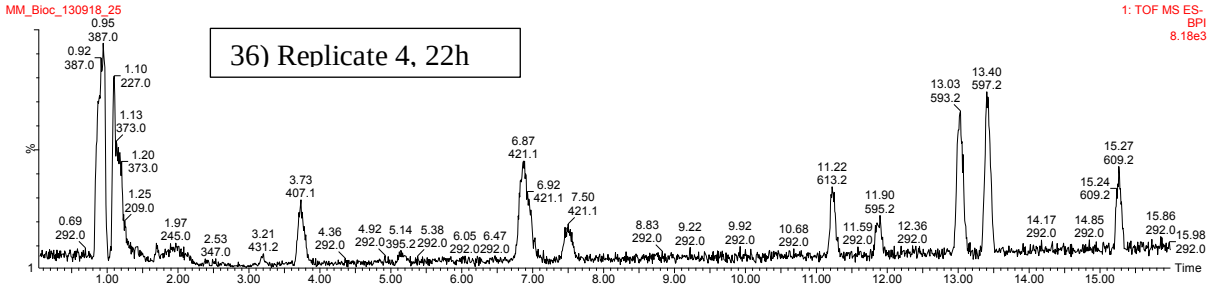


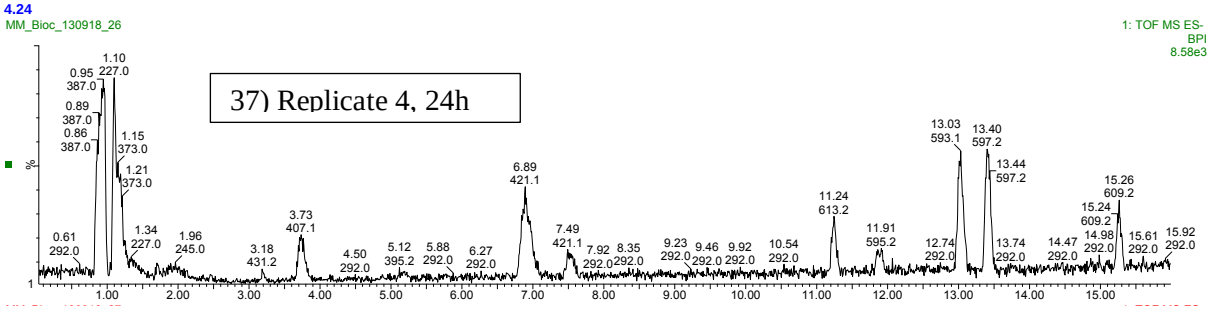


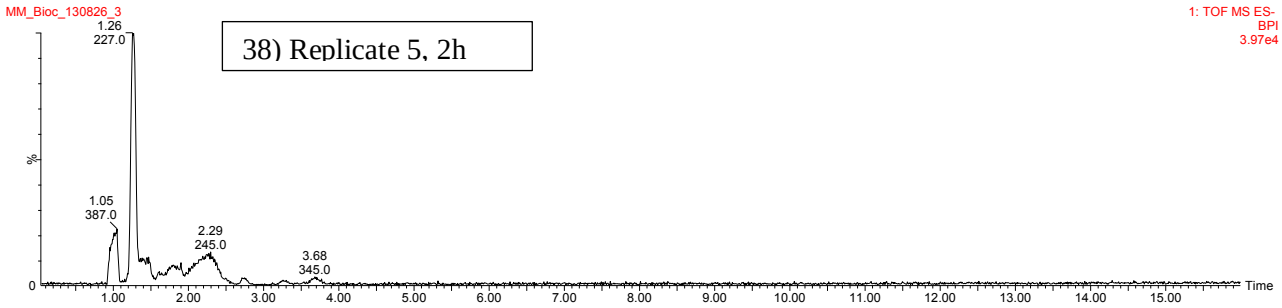


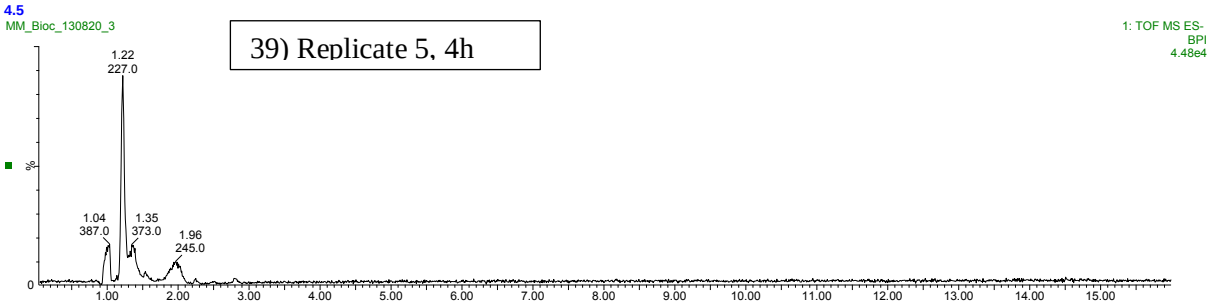


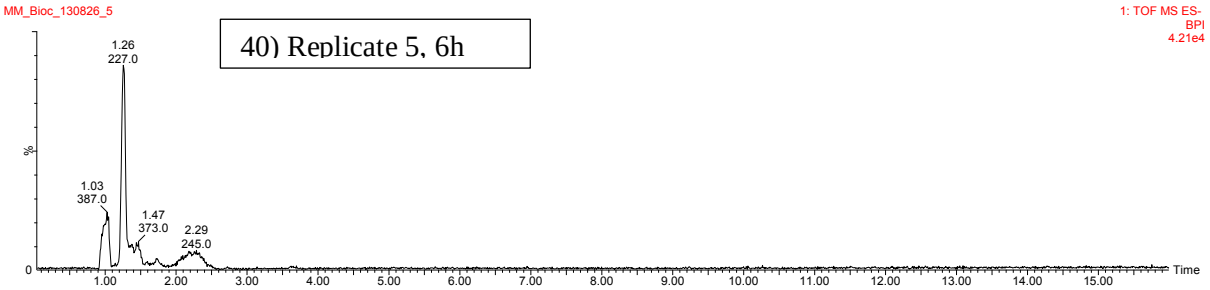


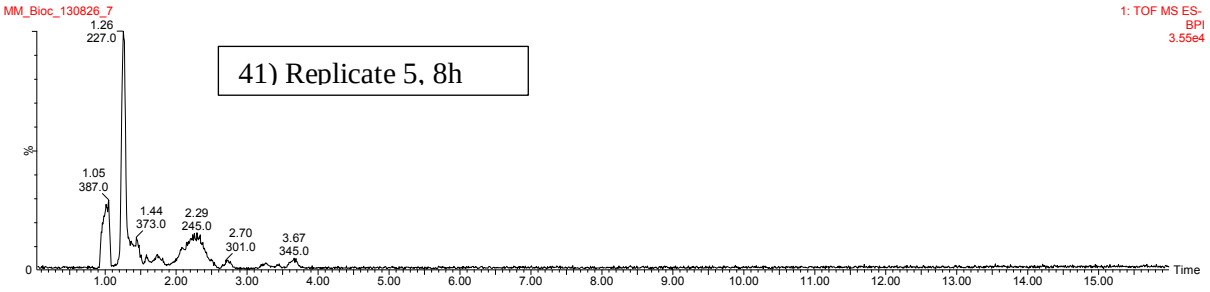


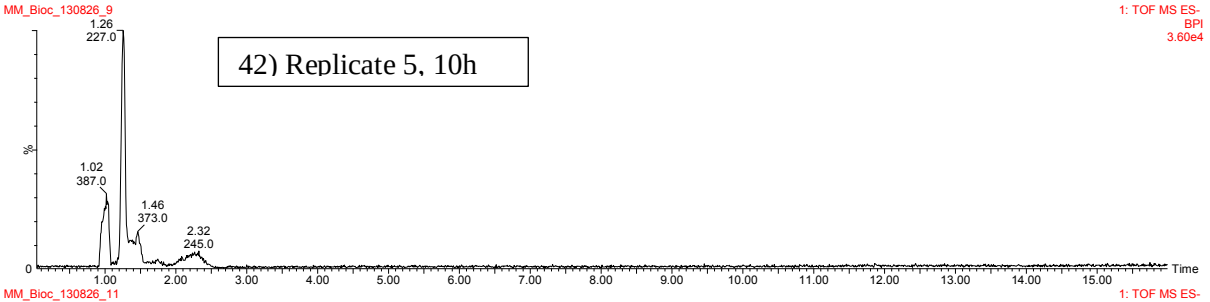


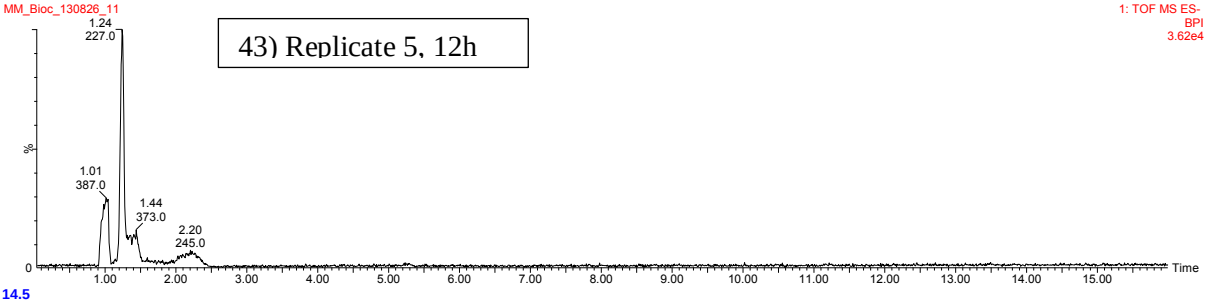


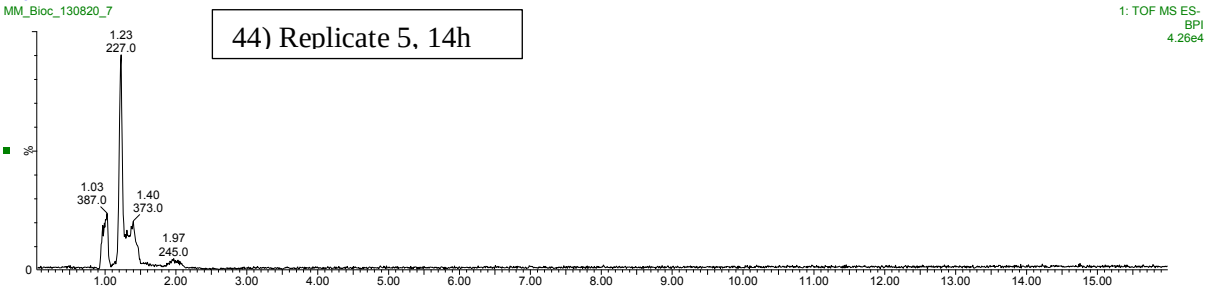


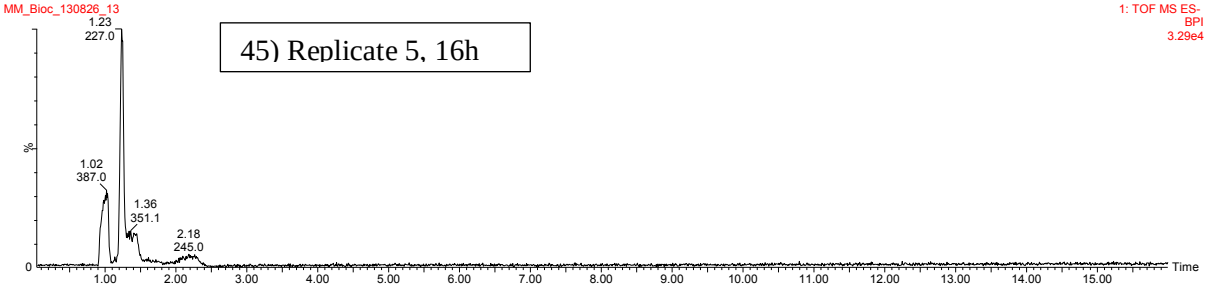


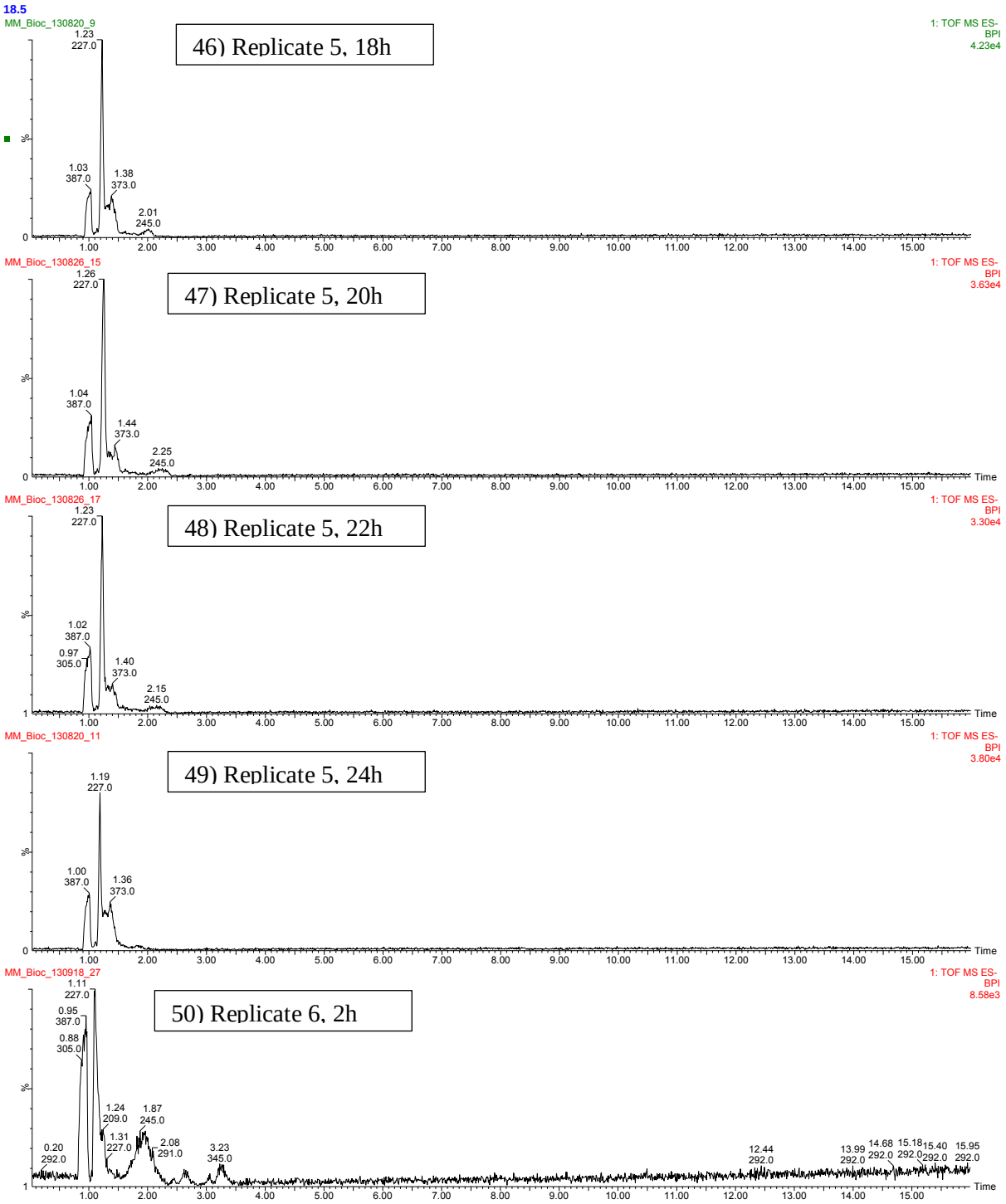


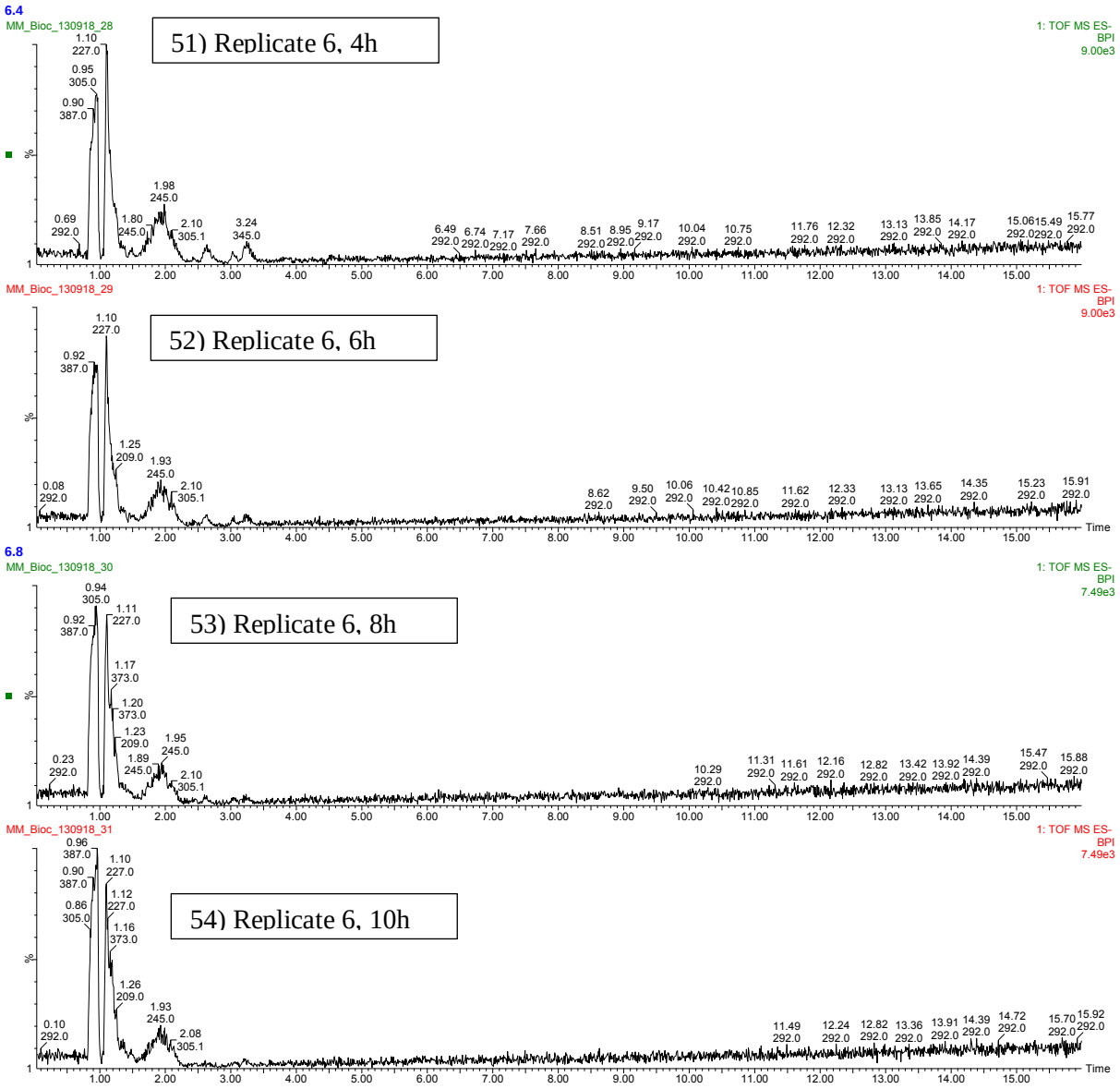


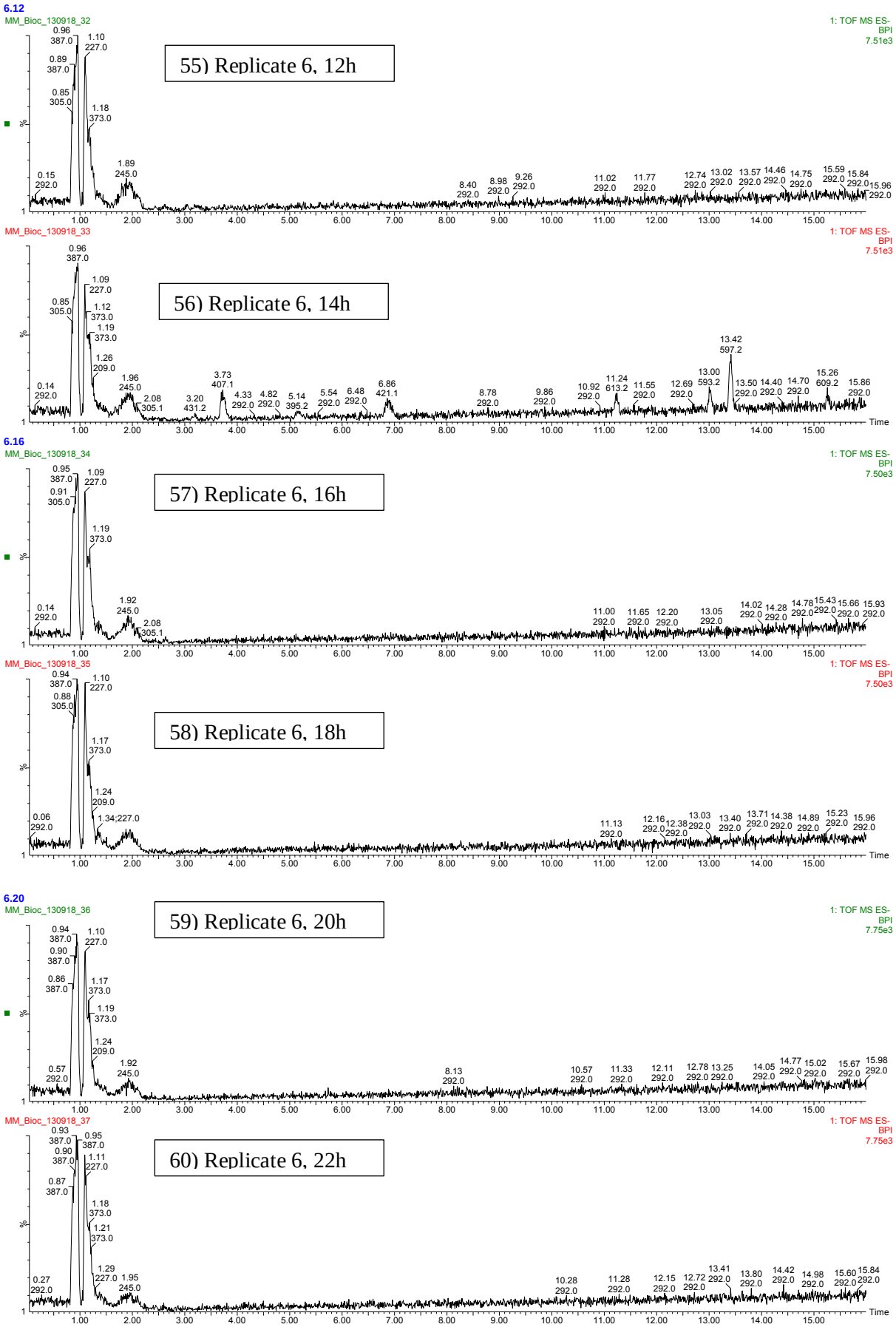


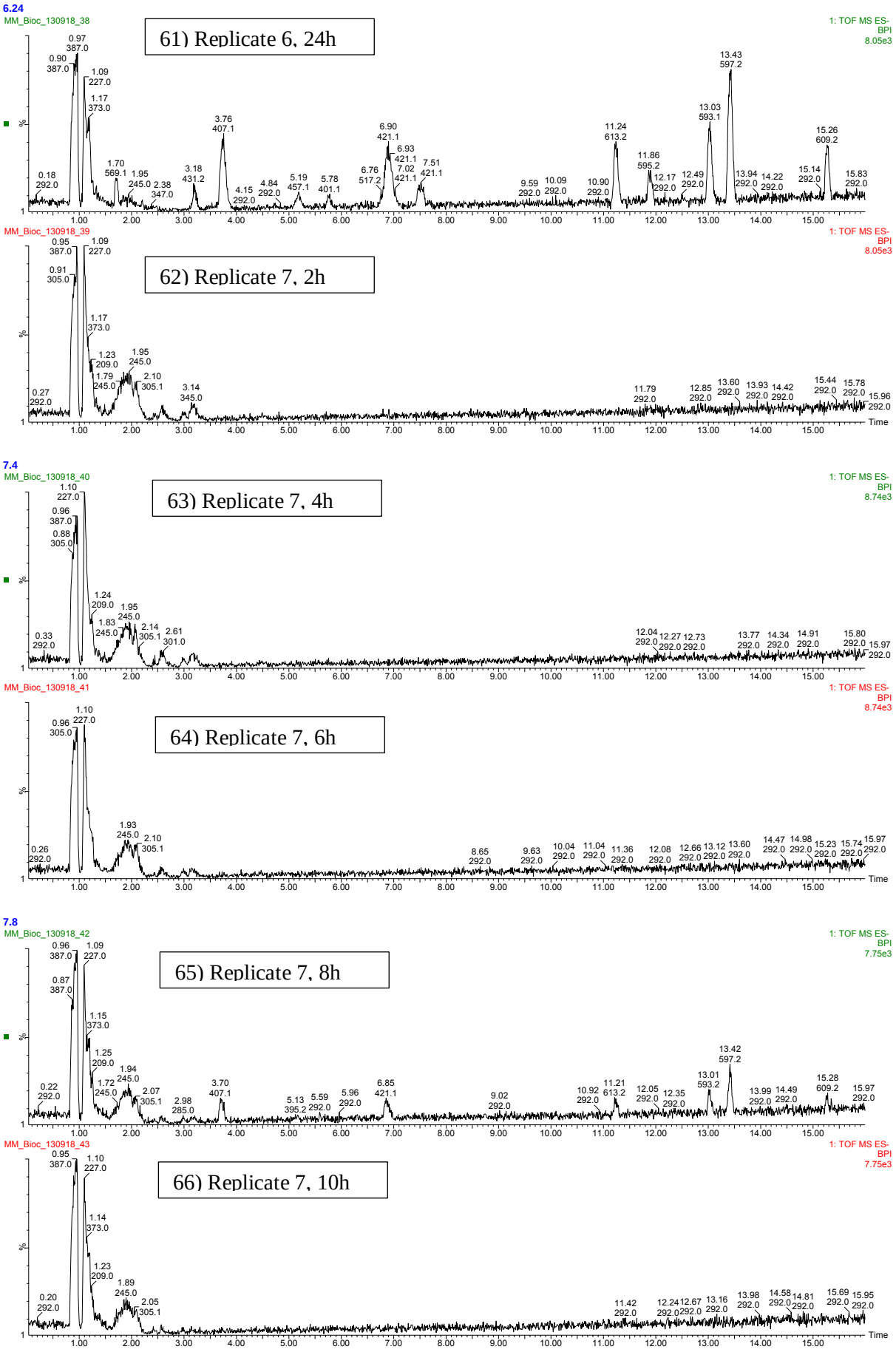


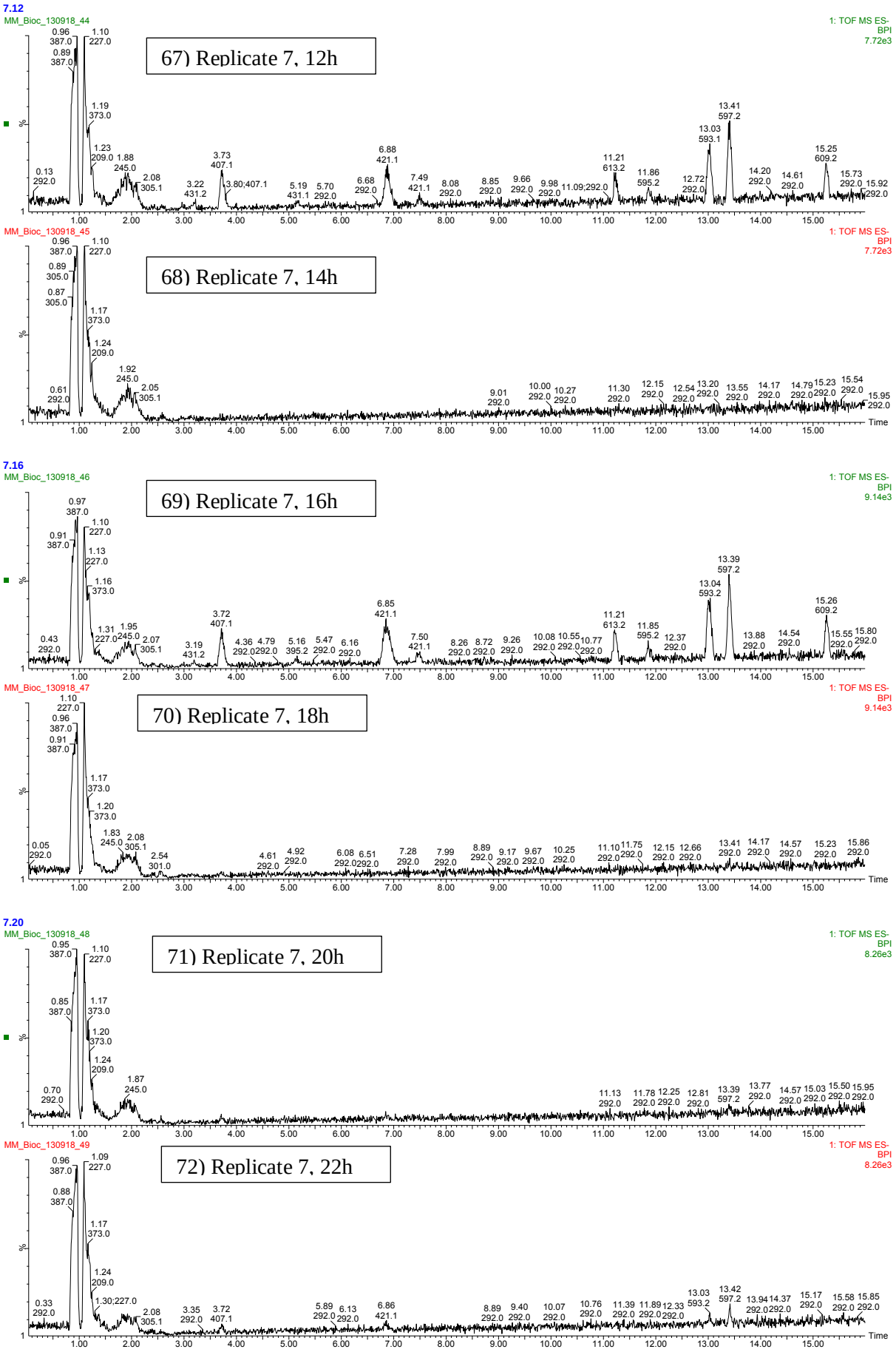


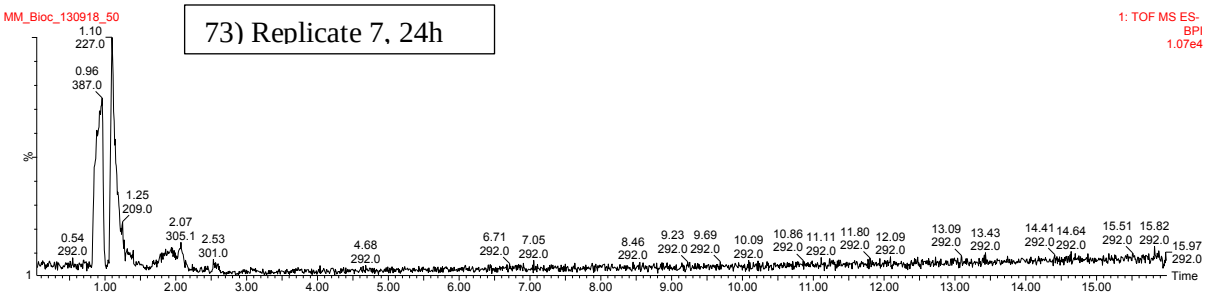






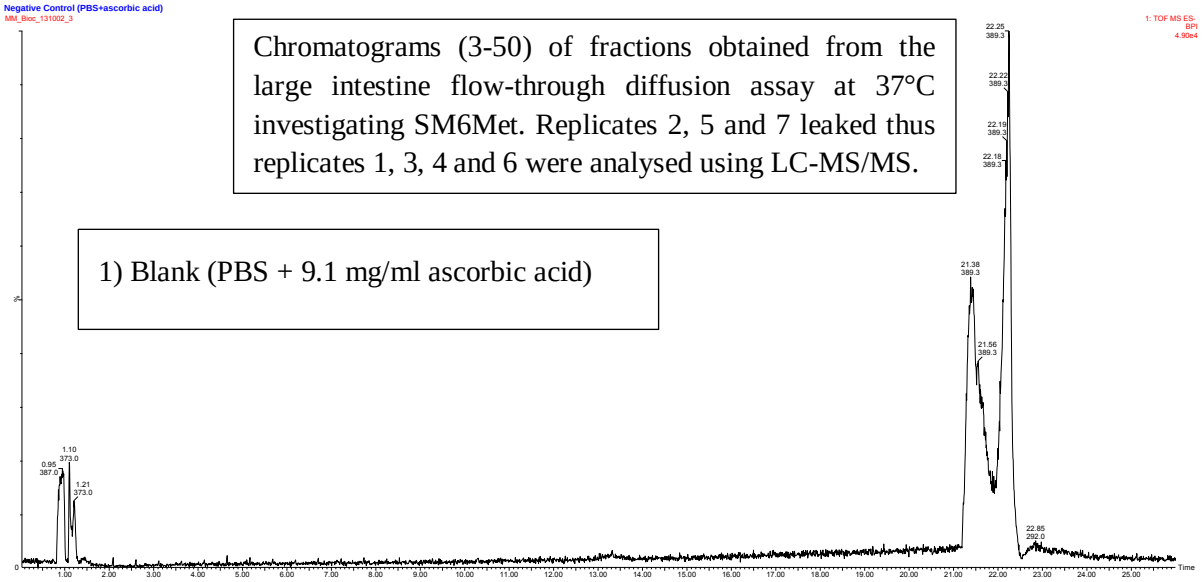


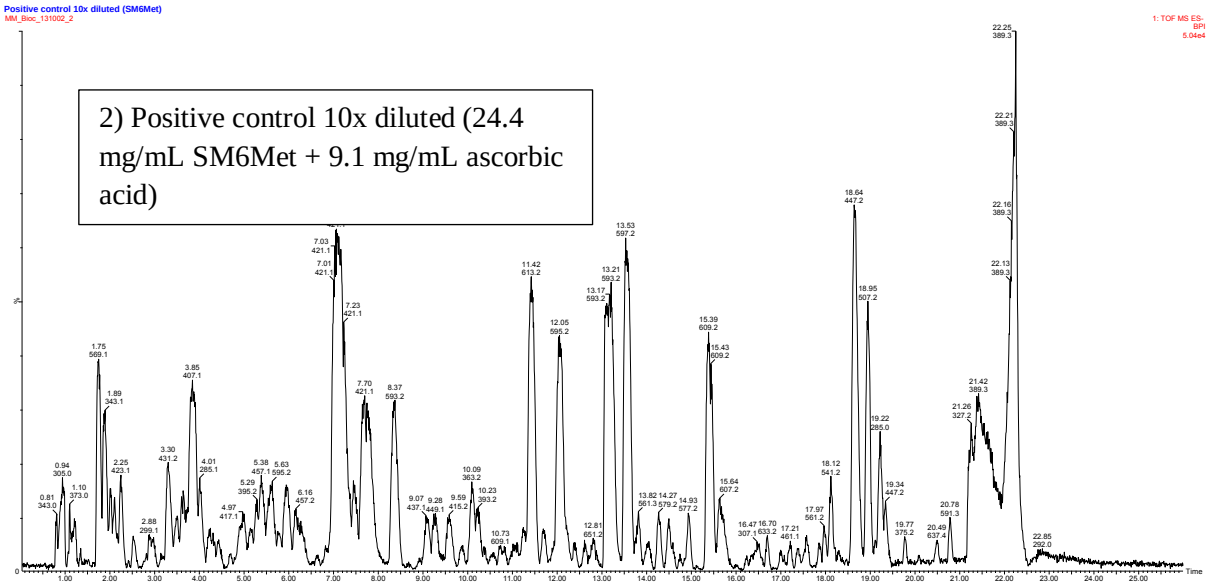


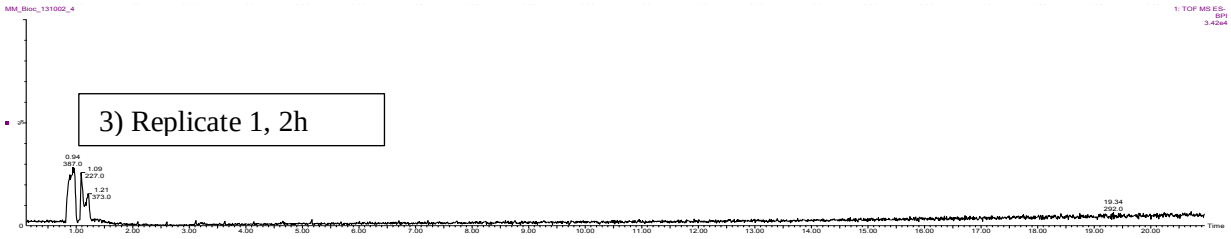


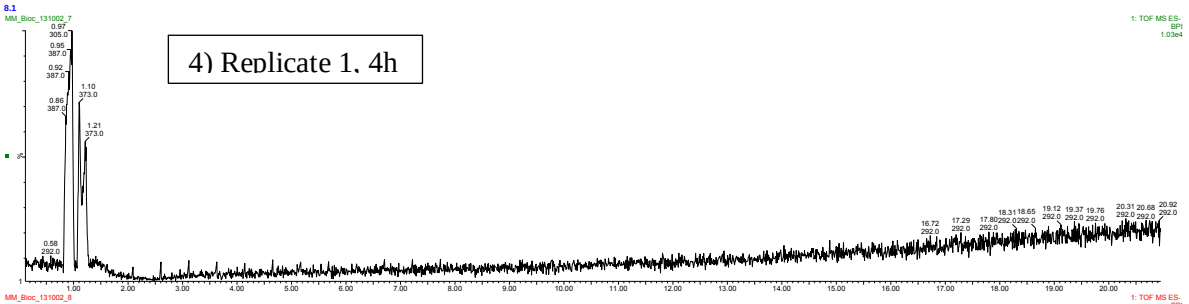
Supplementary Figure 2. Chromatograms obtained from 37°C small intestine flow-through diffusion assay fractions LC-MS/MS analysis of fractions collected every two hours for 24 hours for replicates two and five. Chromatograms 2-73 represent replicates two to seven at the 12, 2 h time intervals for 24 h, represented as % base peak intensity over time. Chromatogram 1 represented the blank (distilled H_2O)

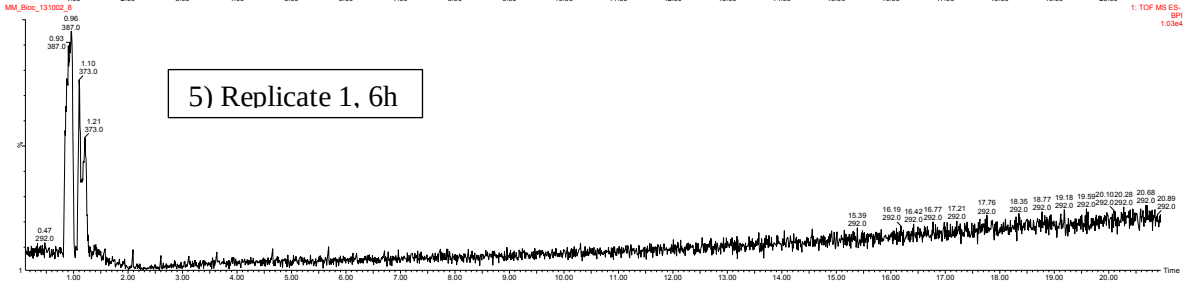
Chromatograms (3-50) of fractions obtained from the large intestine flow-through diffusion assay at 37°C investigating SM6Met. Replicates 2, 5 and 7 leaked thus replicates 1, 3, 4 and 6 were analysed using LC-MS/MS.

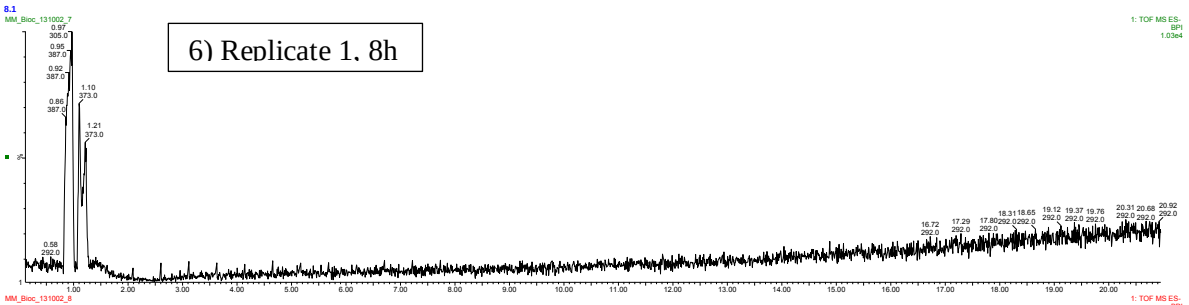


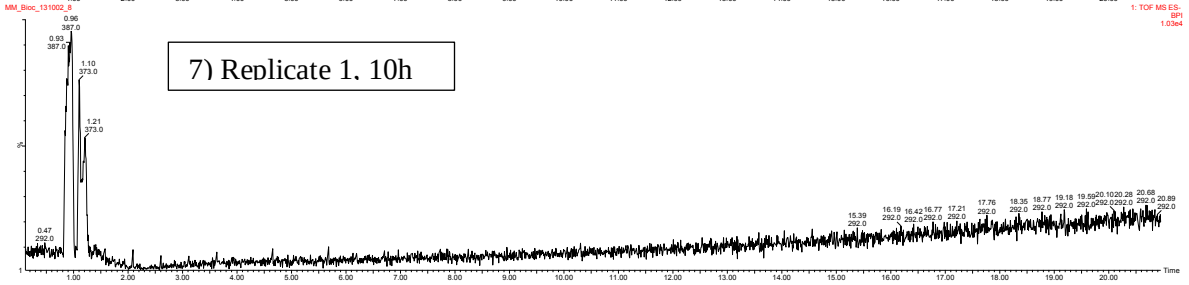


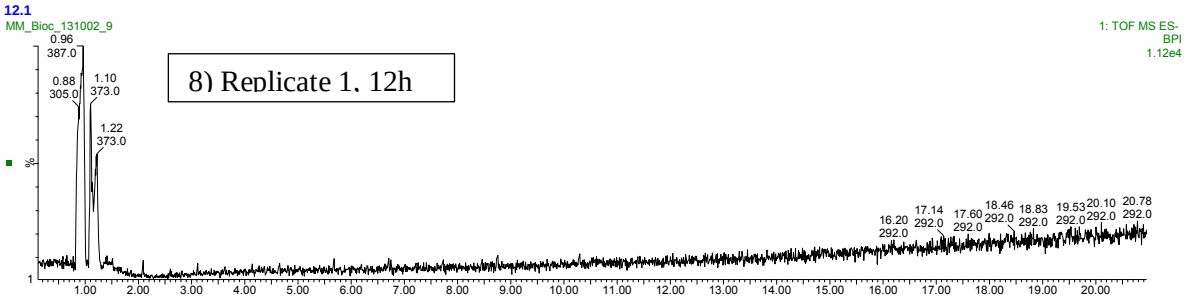


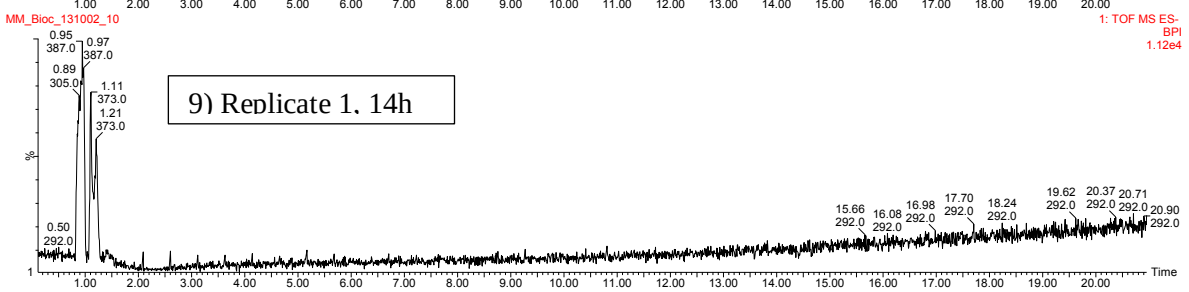


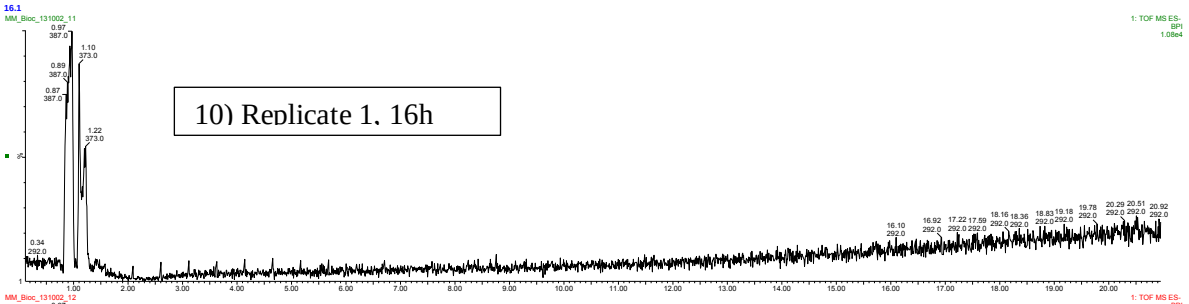


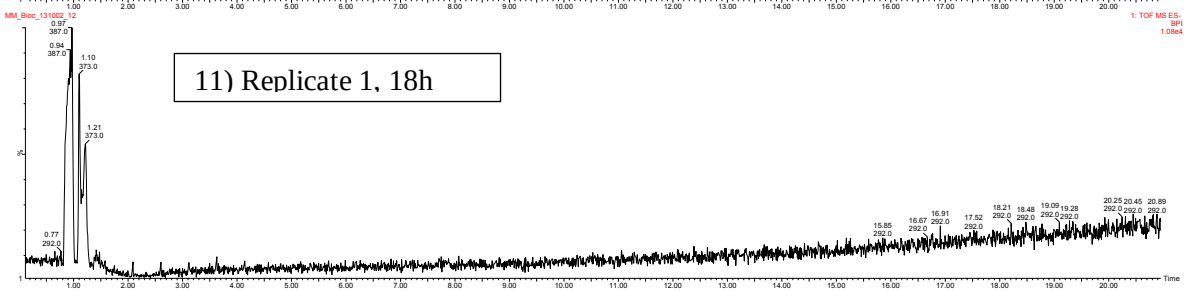


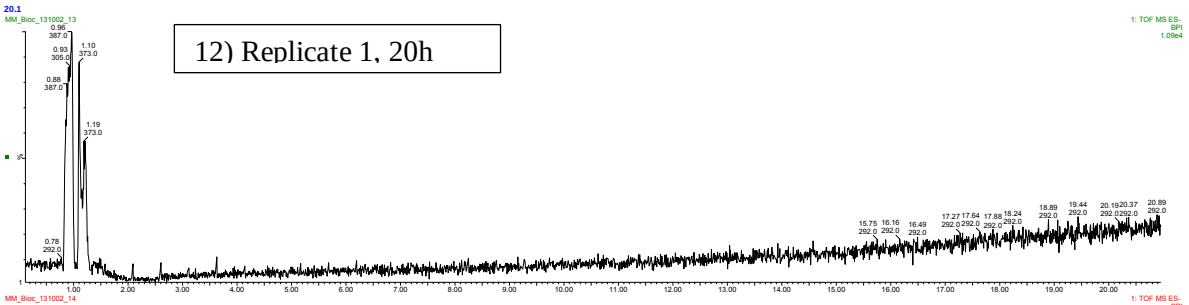


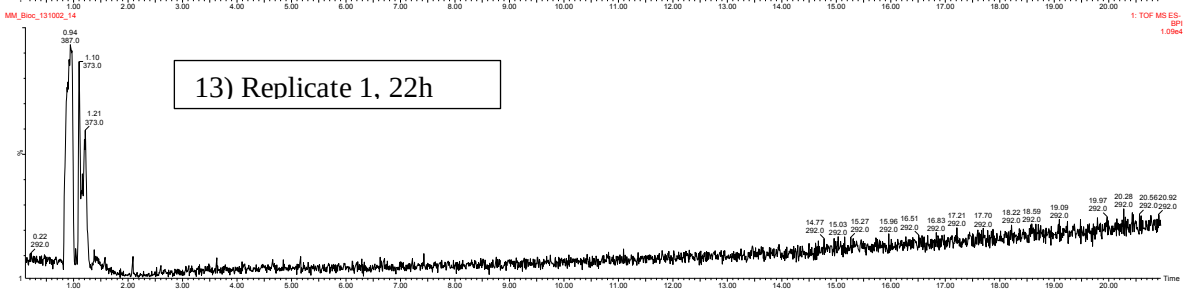


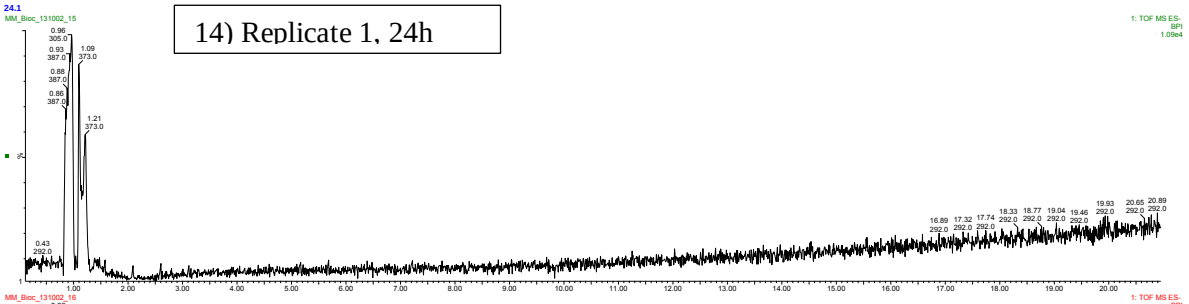


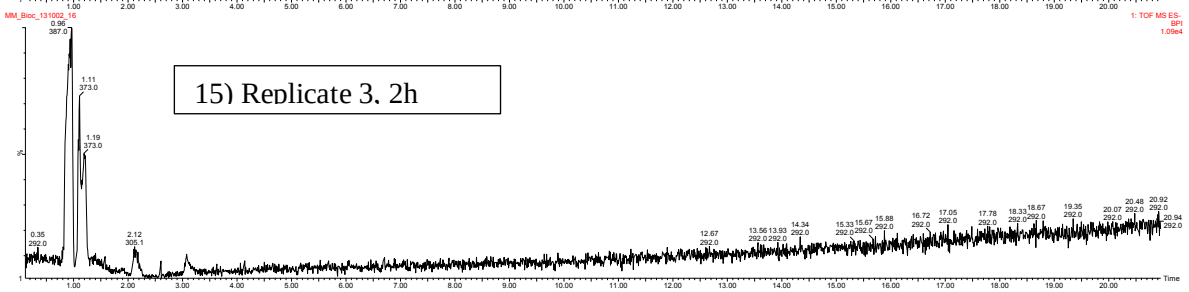


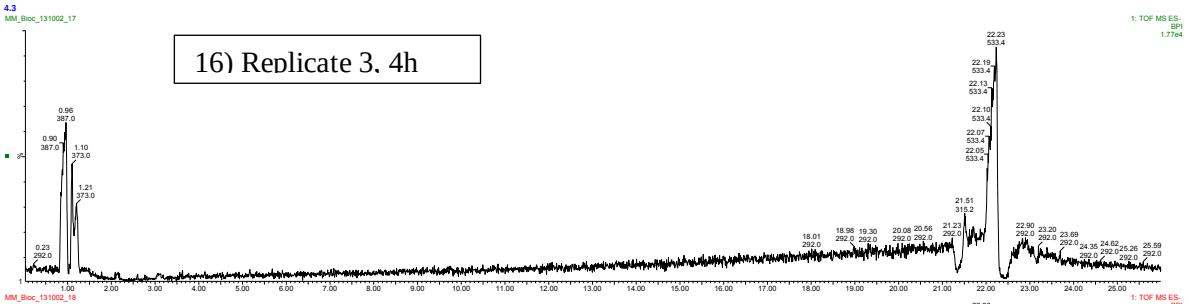












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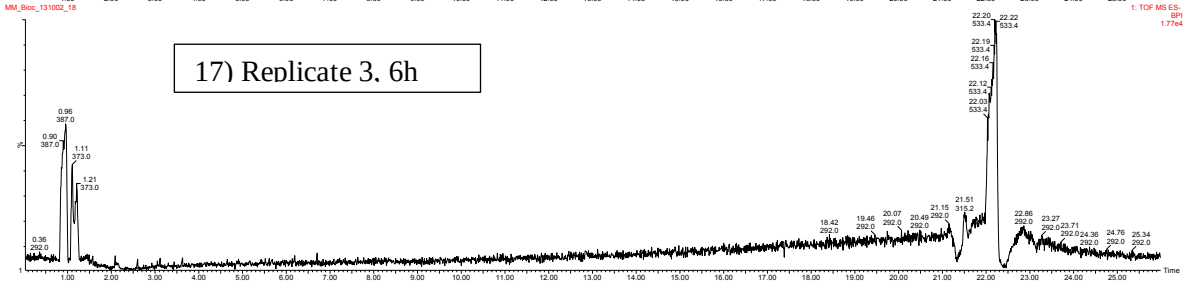

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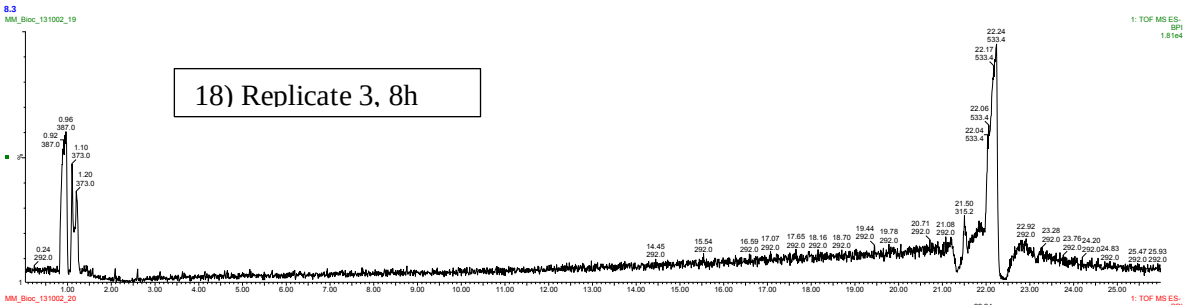

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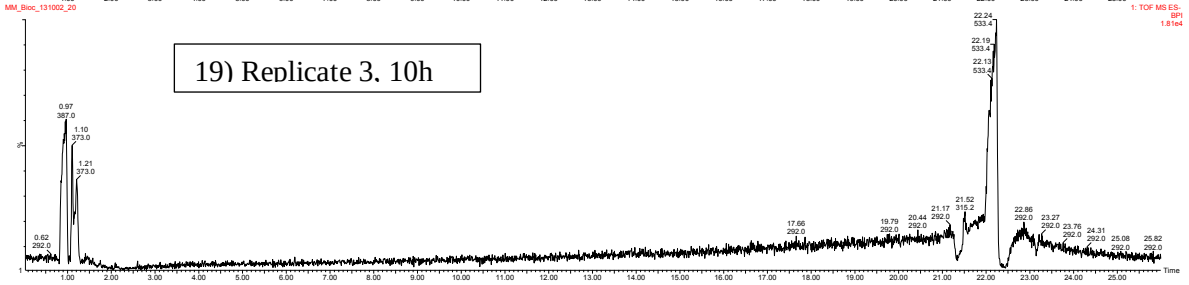

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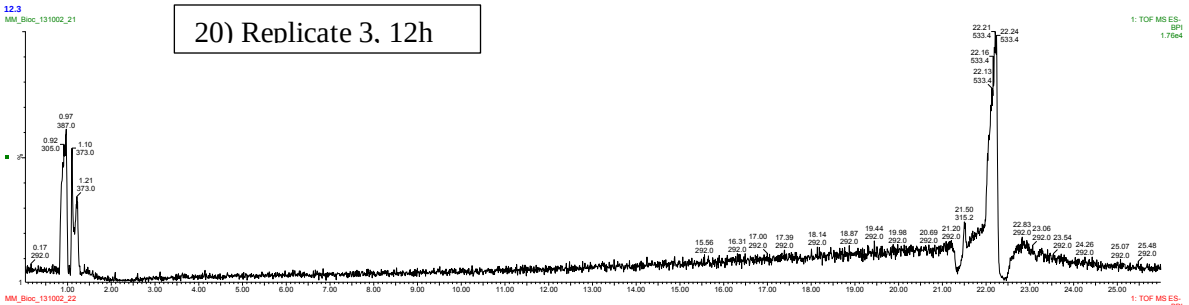

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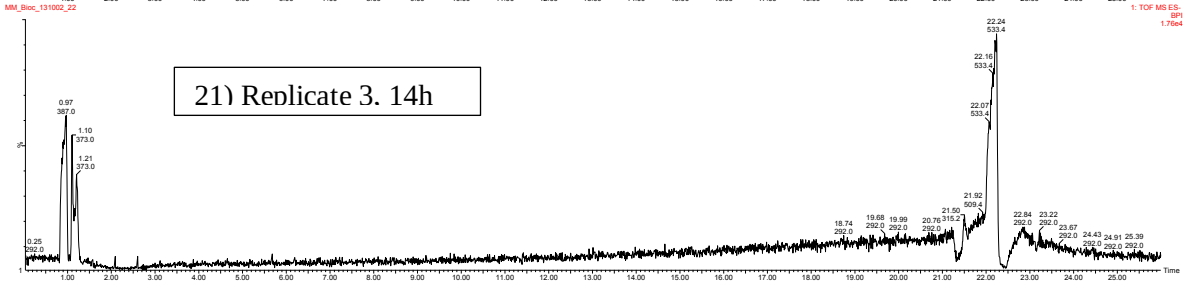

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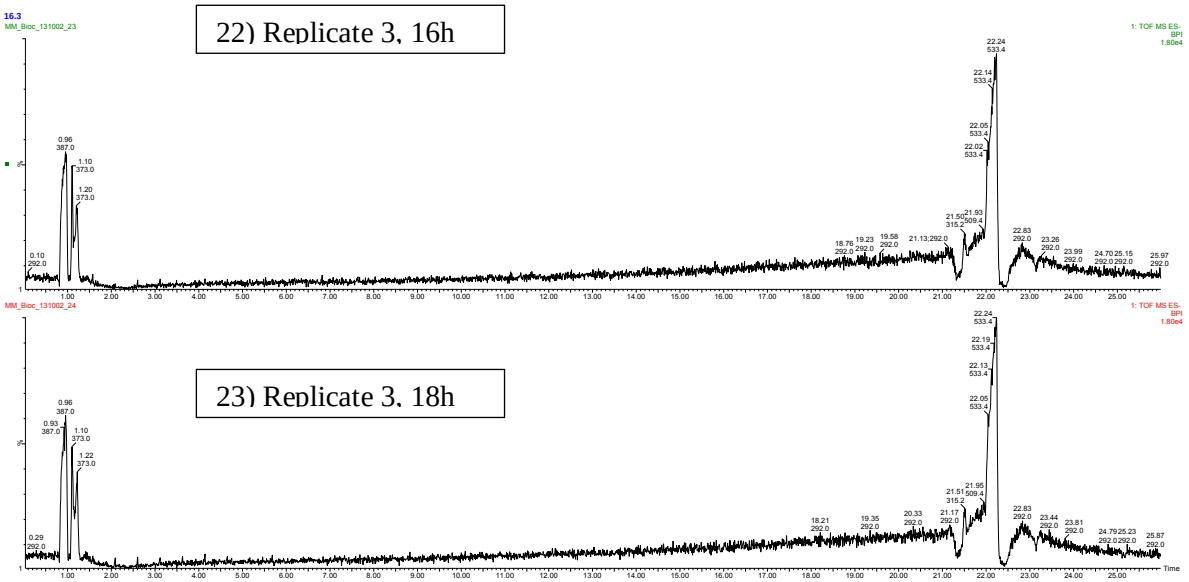
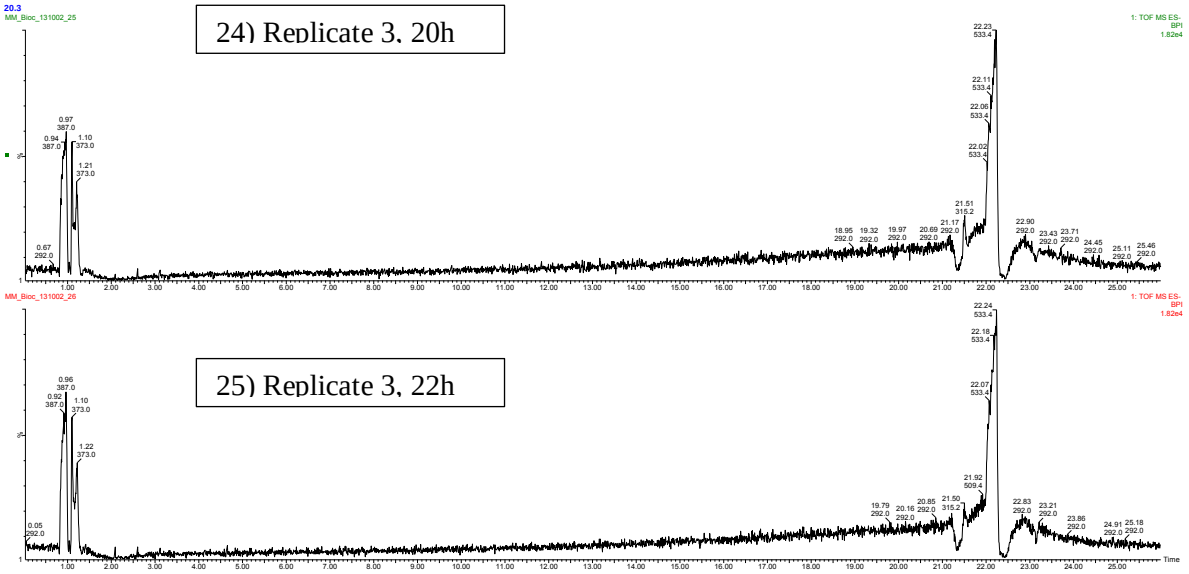
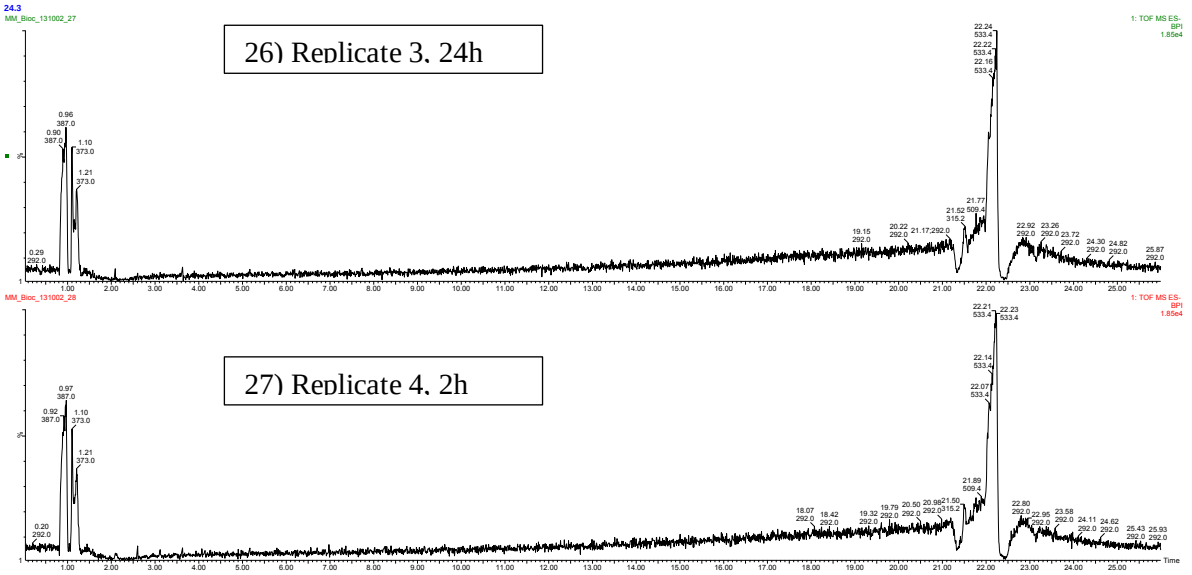
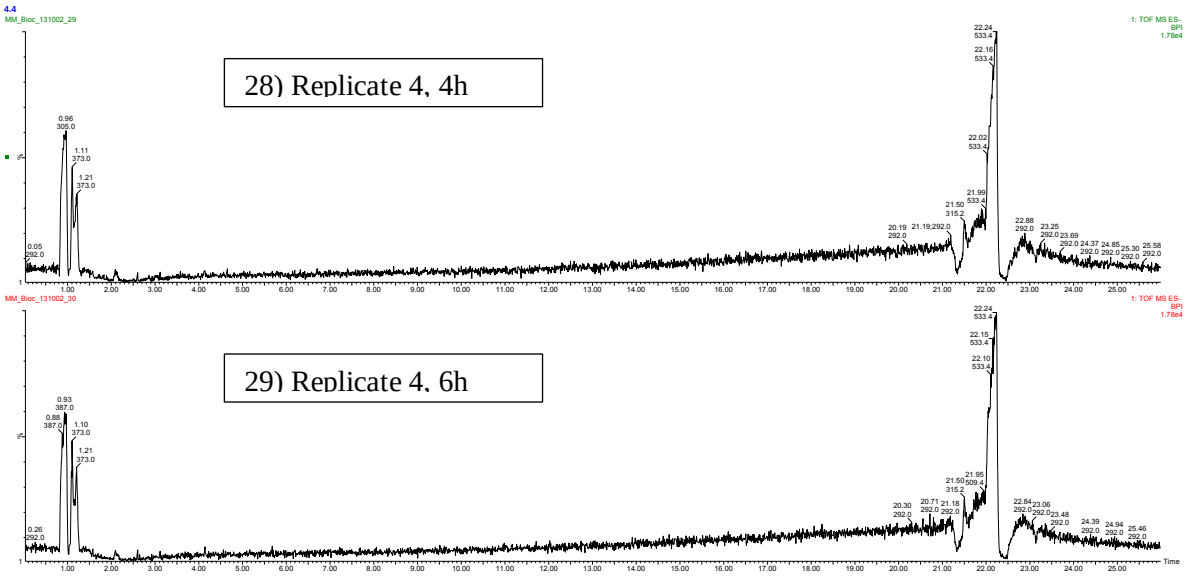
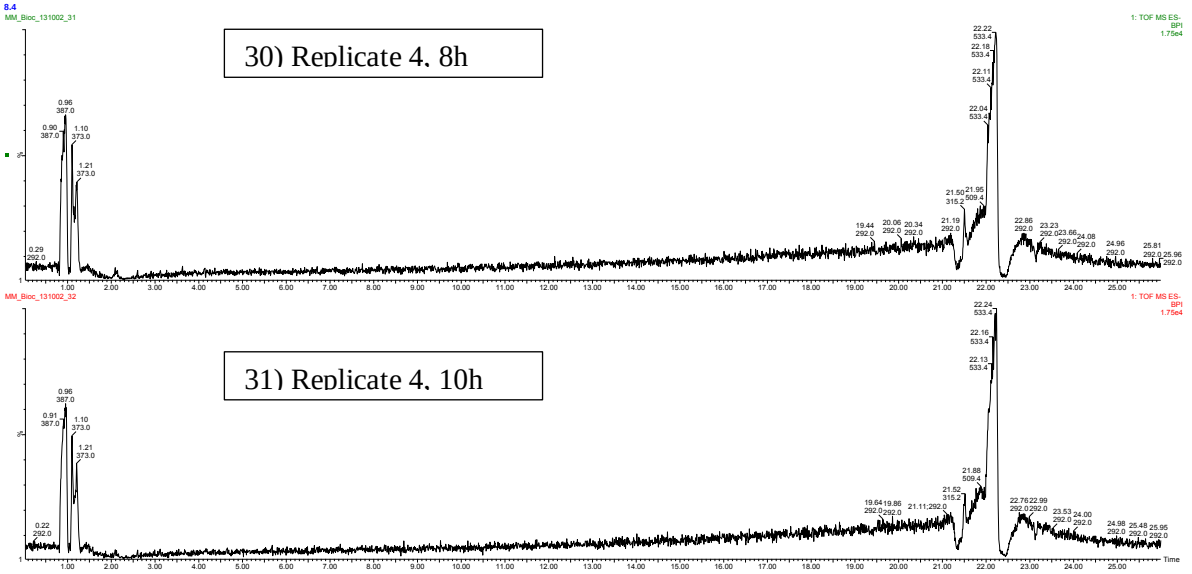
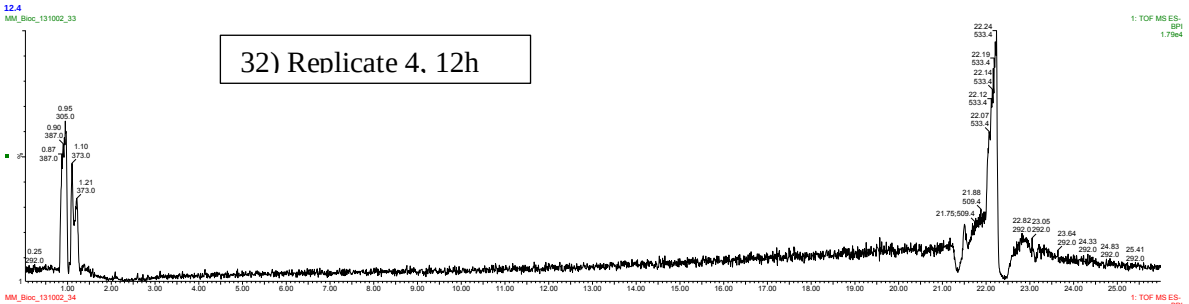


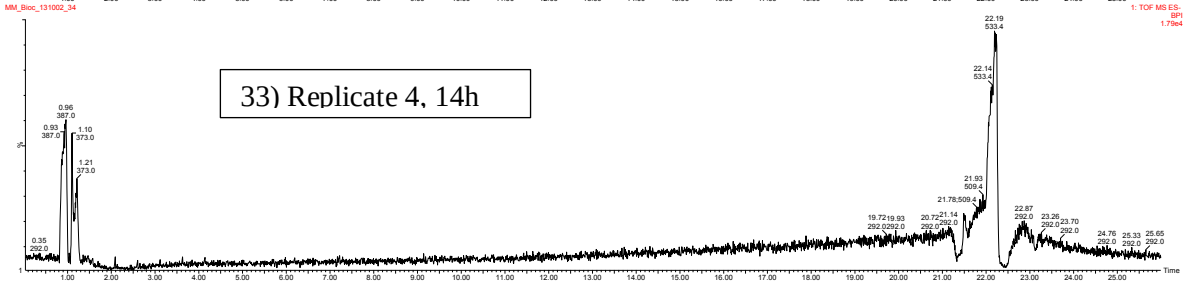


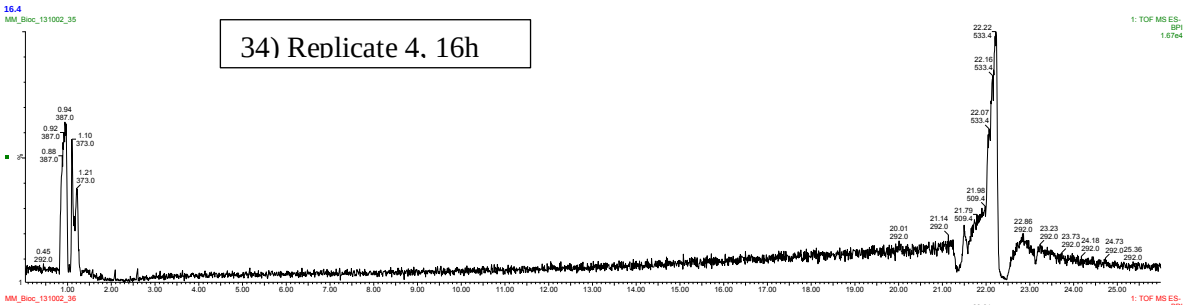


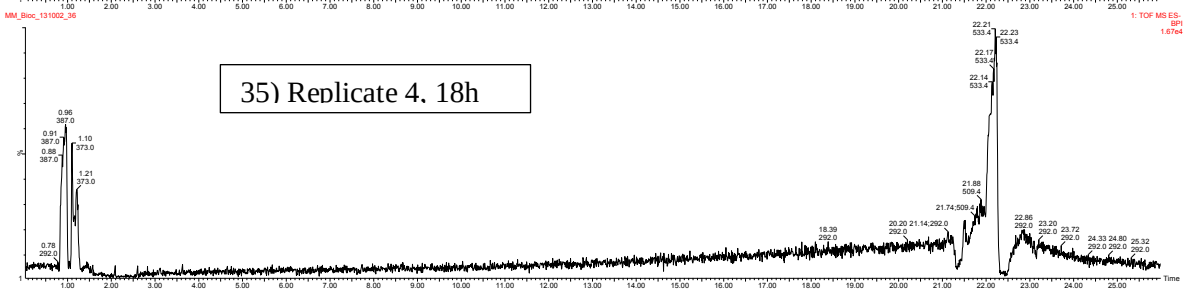


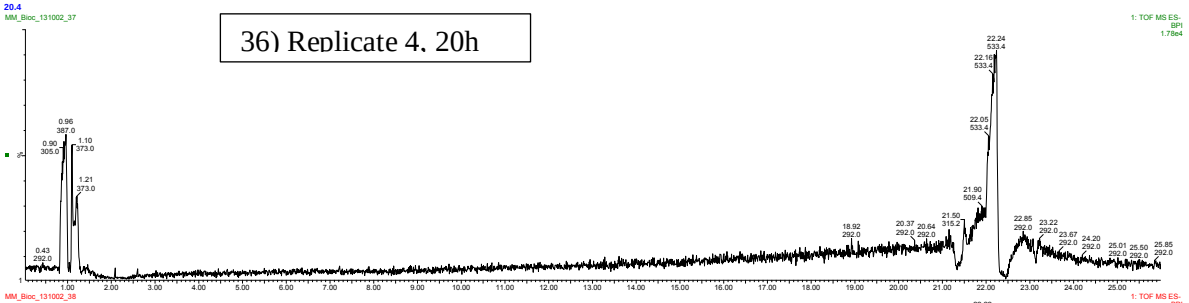


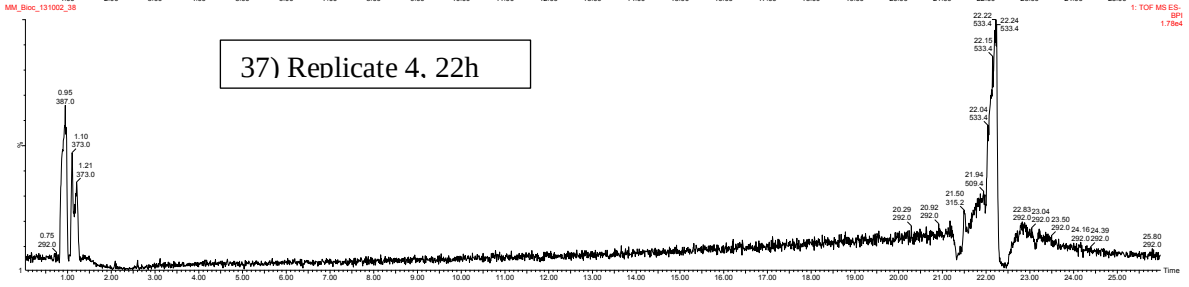


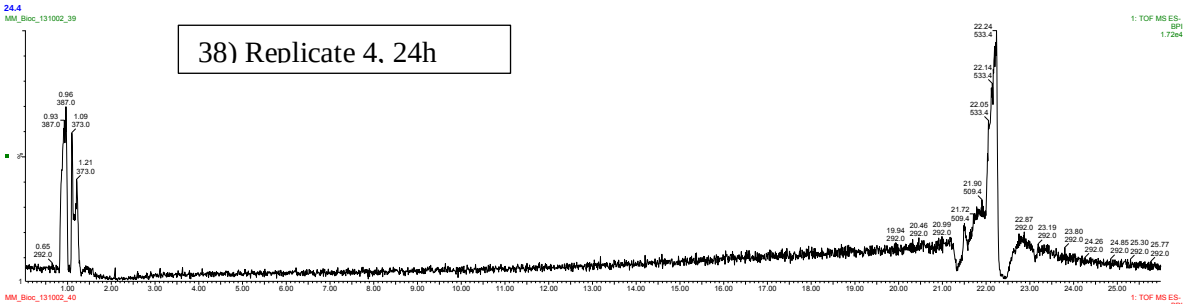


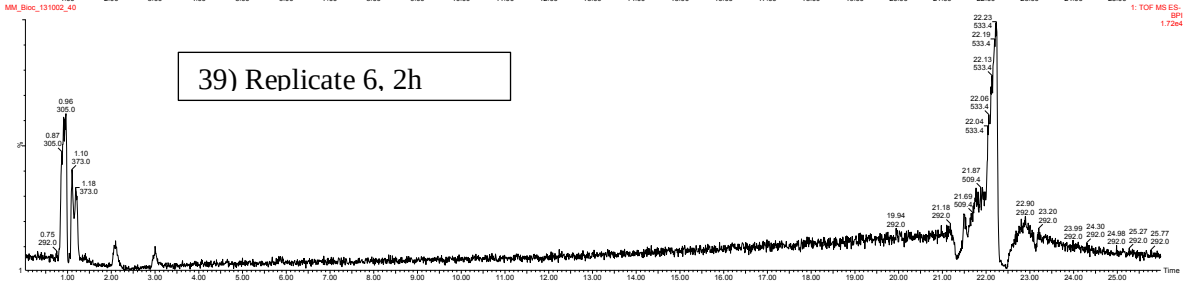


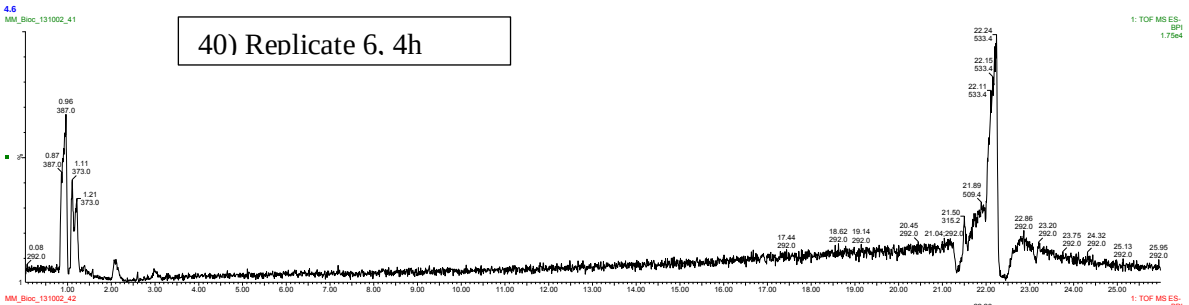


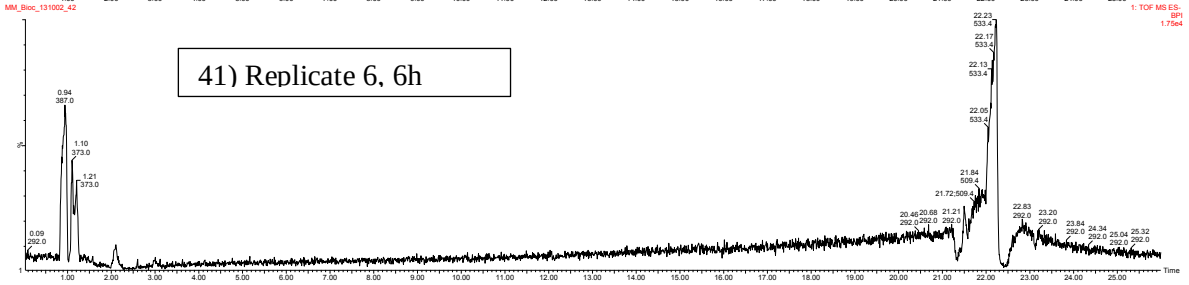


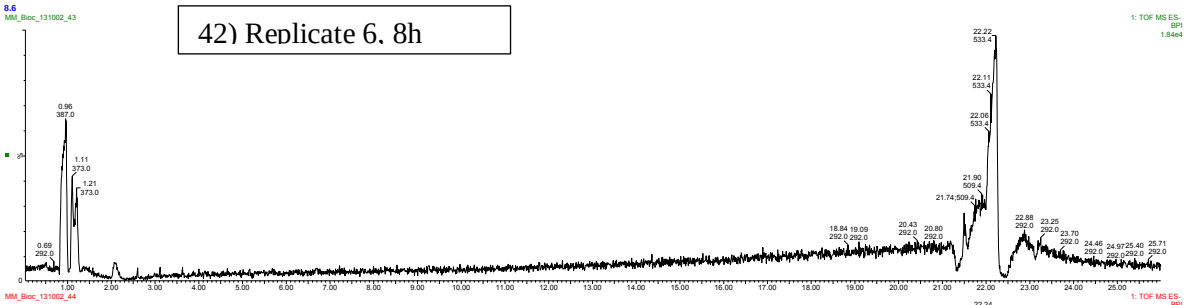


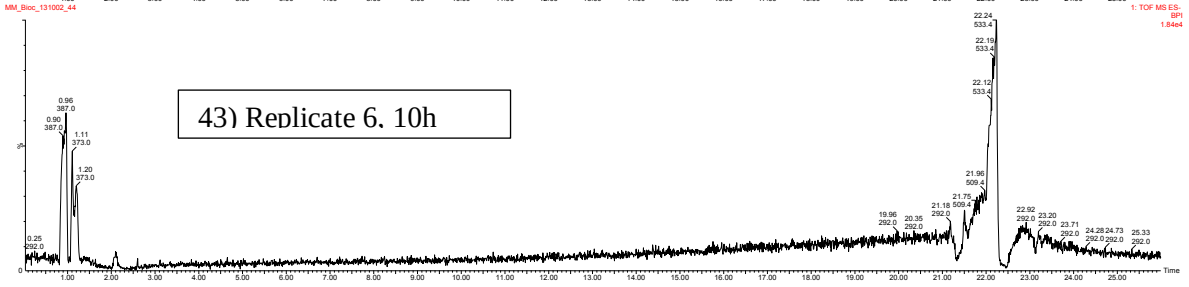


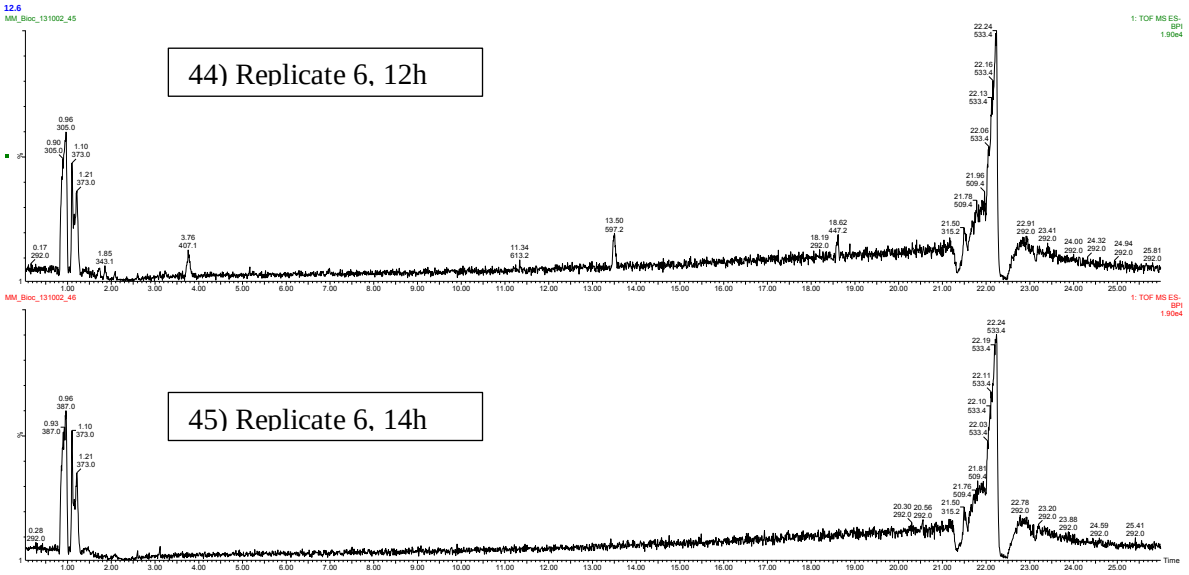


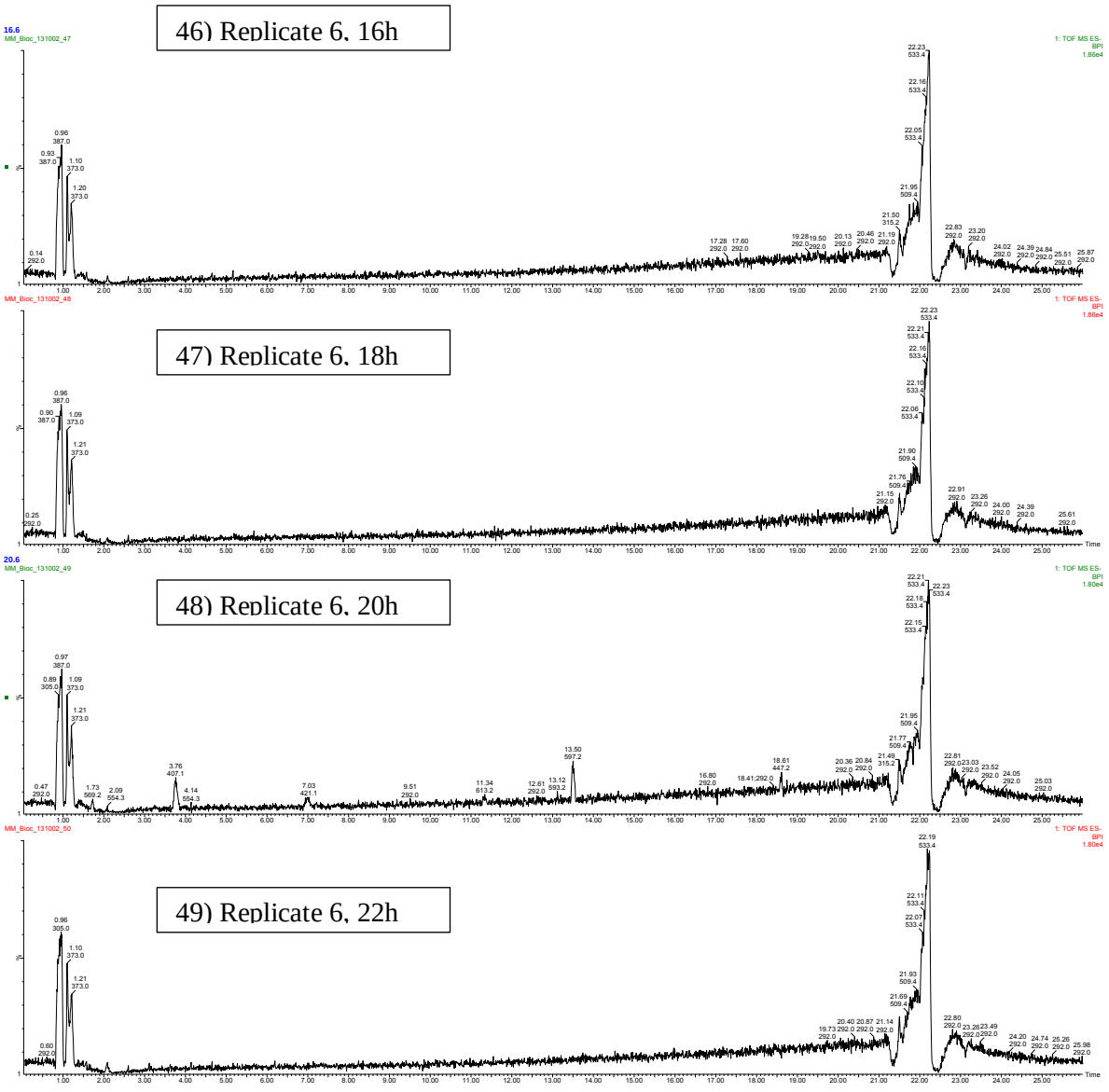


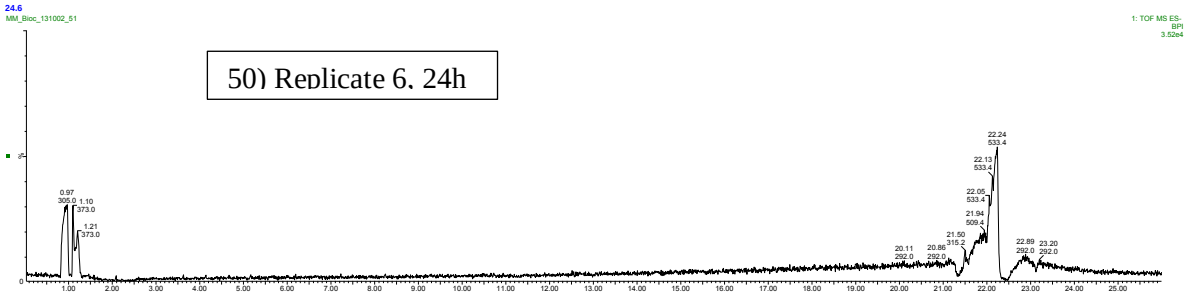




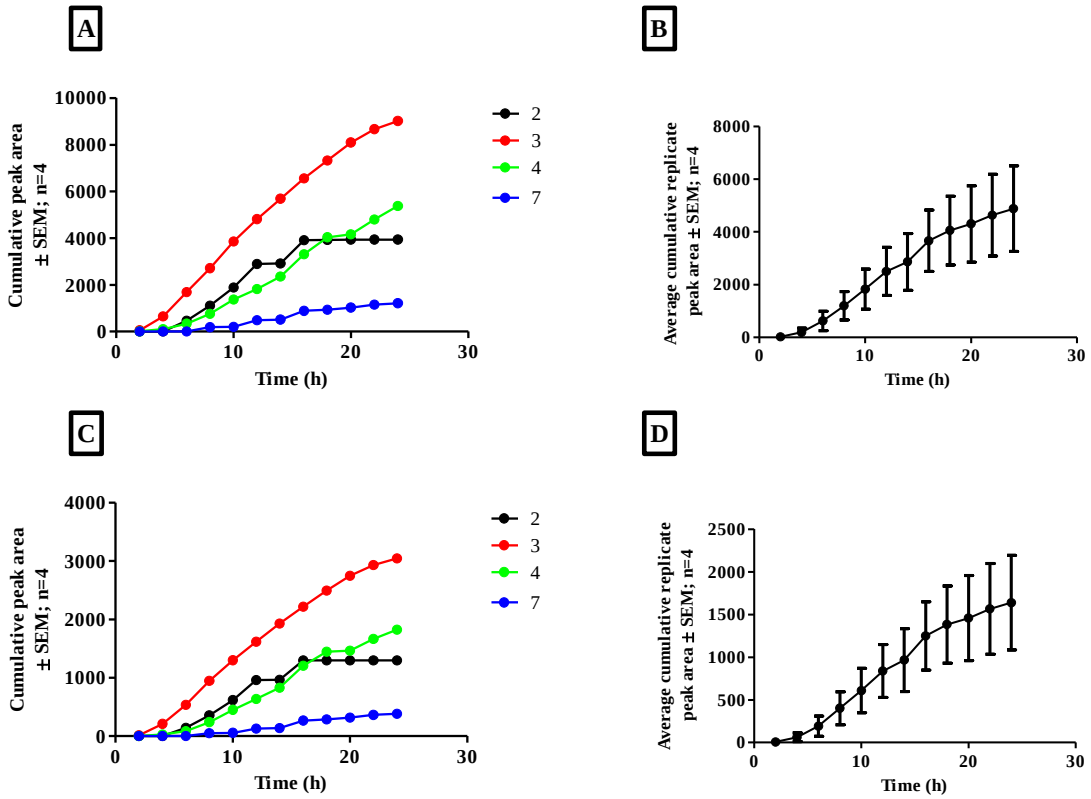




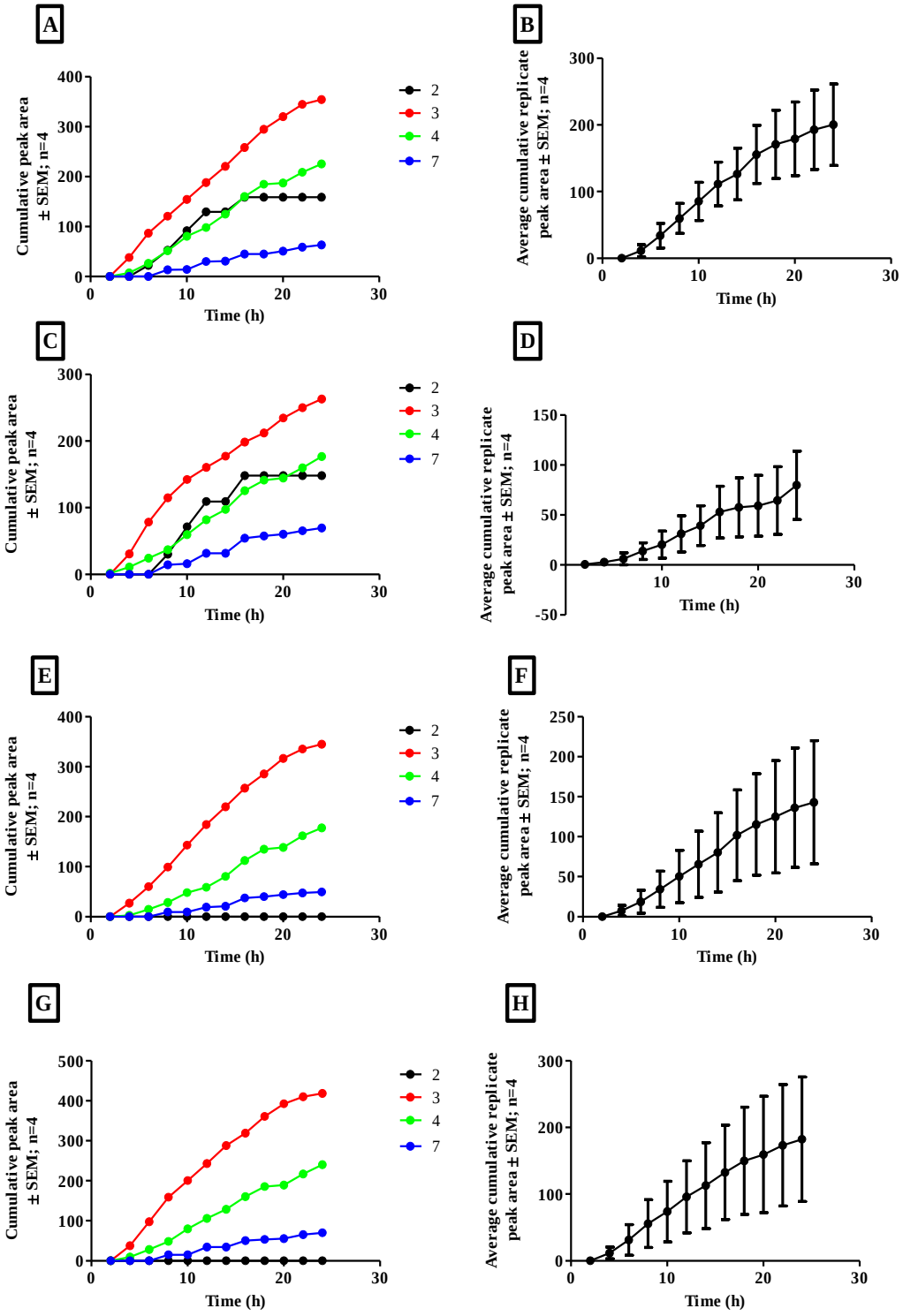


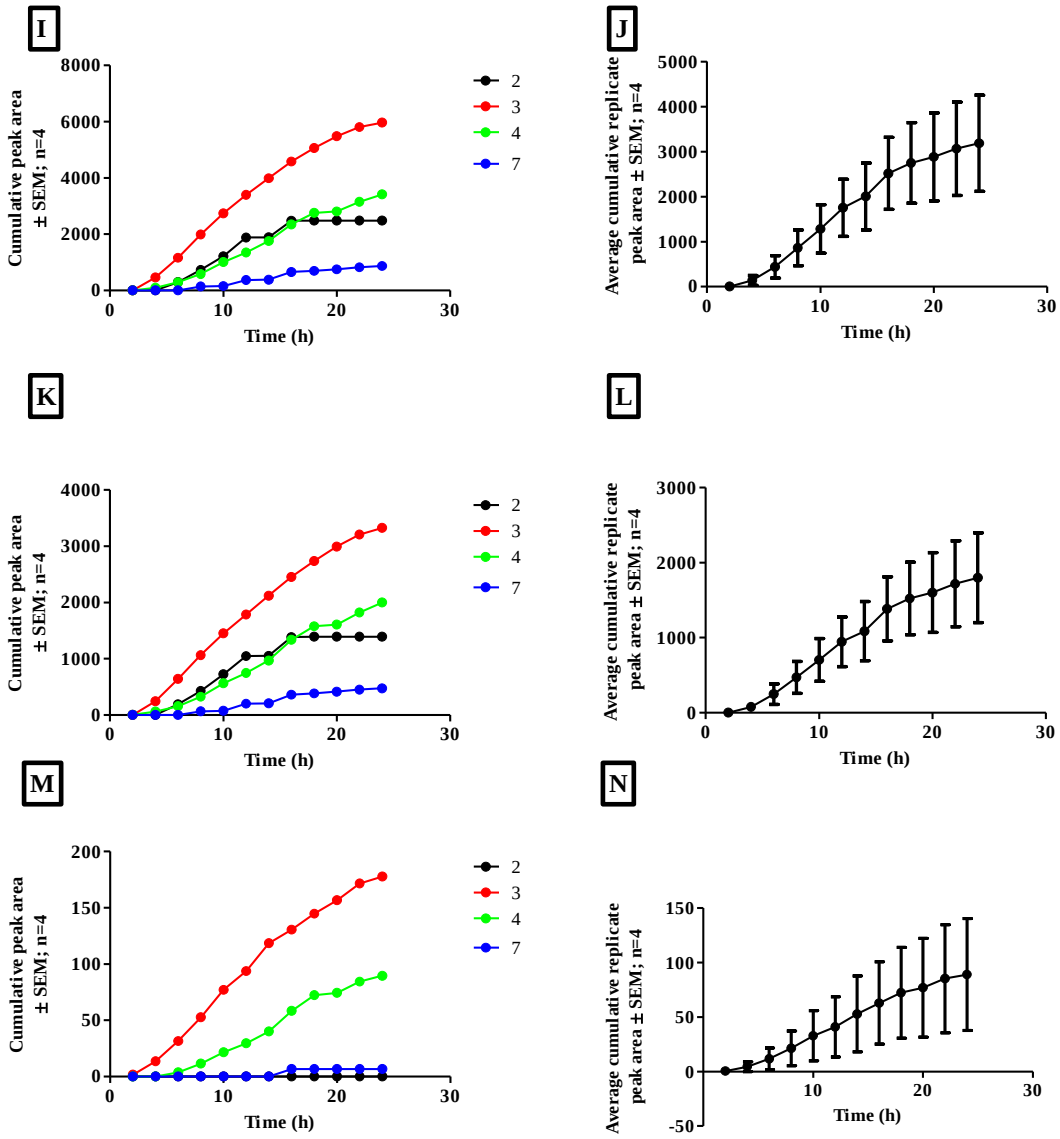


Supplementary Figure 3. Chromatograms obtained from 37°C large intestine flow-through diffusion assay fractions LC-MS/MS analysis of fractions collected every two hours for 24 hours for replicates two and five. Chromatograms 3-50 represent replicates one, three, four and six at the 12 2 h time intervals for 24 h, represented as % base peak intensity over time. Chromatogram 1 represented the blank (PBS with 9.1 mg/mL ascorbic acid) and chromatogram 2 represented the positive control (10x diluted).

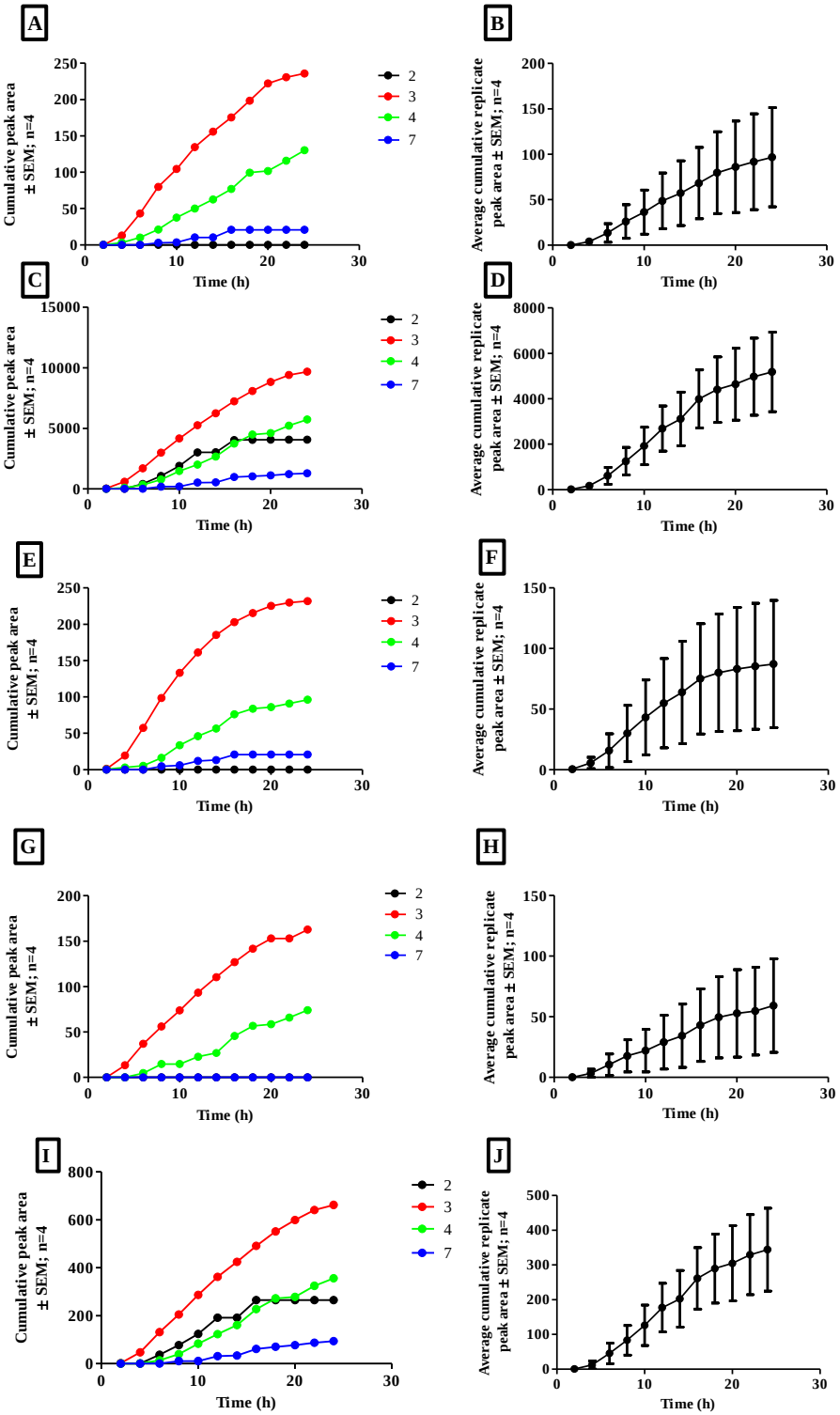


Supplementary Figure 4 Diffusion analysis of the xanthones originating from SM6Met via porcine small intestine by means of comparing accumulated polyphenol small intestinal cross-over. The permeability assay was performed at 37°C, over 24 h, using the flow-through diffusion system [22], where after fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) individual replicate cumulative peak area of mangiferin; B) average replicate cumulative peak area of mangiferin; C) individual replicate cumulative peak area of isomangiferin; D) average replicate cumulative peak area of isomangiferin. Replicates five and six where excluded in all instances.

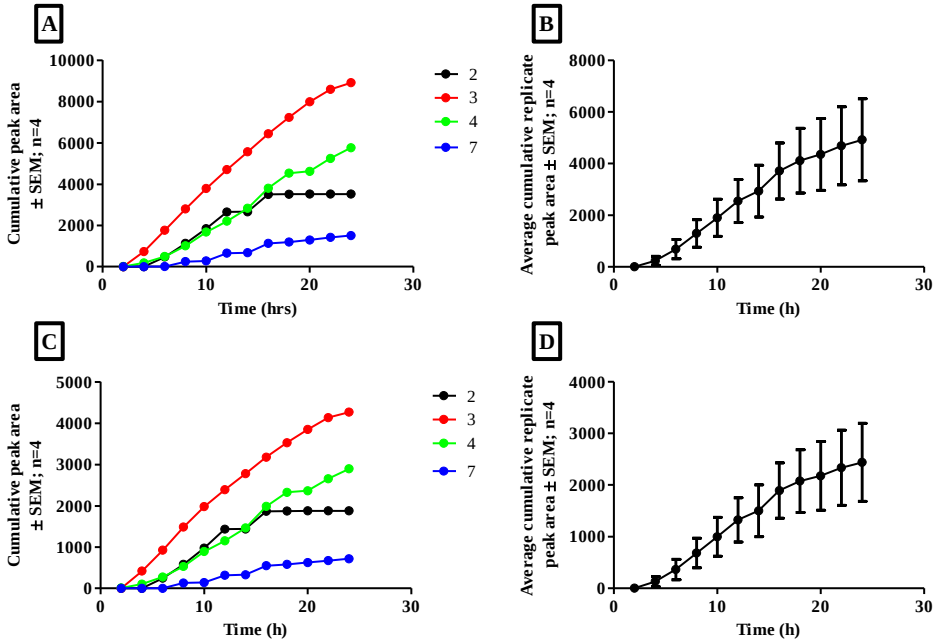




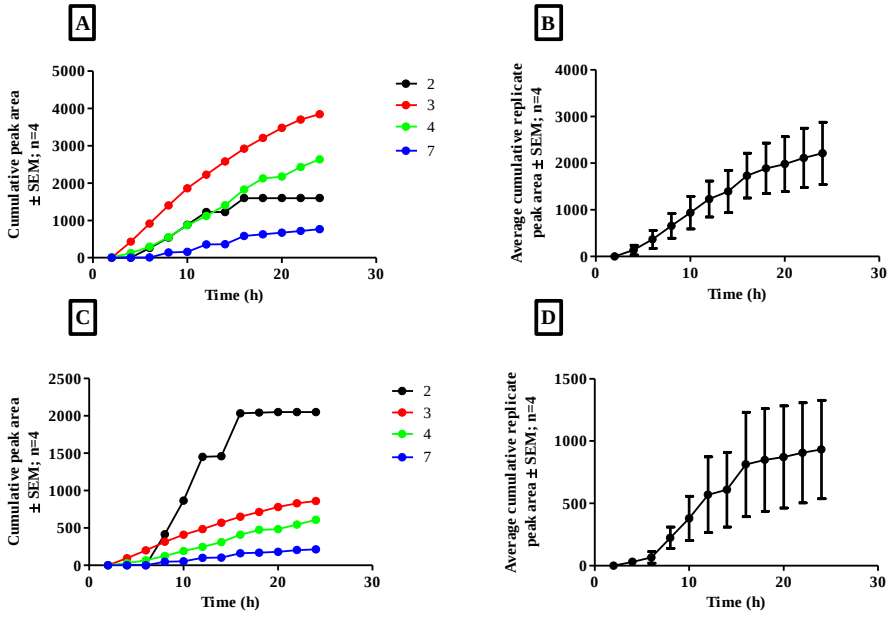
Supplementary Figure 5 Diffusion analysis of the flavanones originating from SM6Met via porcine small intestine by means of comparing accumulated polyphenol small intestinal cross-over. The permeability assay was performed at 37°C, over 24 h, using the flow-through diffusion system [16], where after fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) individual replicate cumulative peak area of (*S*)-eriodictyol-di-*C*-hexoside; B) average replicate cumulative peak area of (*S*)-eriodictyol-di-*C*-hexoside; C) individual replicate cumulative peak area of (*R*)-eriodictyol-di-*C*-hexoside; D) average replicate cumulative peak area of (*R*)-eriodictyol-di-*C*-hexoside; E) individual replicate cumulative peak area of naringenin-*O*-dihexoside; F) average replicate cumulative peak area of to naringenin-*O*-dihexoside; G) individual replicate cumulative peak area of naringenin-di-*C*-hexoside; H) average replicate cumulative peak area of naringenin-di-*C*-hexoside; I) individual replicate cumulative peak area of hesperidin; J) average replicate cumulative peak area of hesperidin; K) individual replicate cumulative peak area of eriocitrin; L) average replicate cumulative peak area of eriocitrin; M) individual replicate cumulative peak area of eriodictyol-*O*-glucoside; N) average replicate cumulative peak area of eriodictyol-*O*-glucoside diffusion across the small intestine over time. Replicates five and six where excluded in all instances.



Supplementary Figure 6 Diffusion analysis of the flavones originating from SM6Met via porcine small intestine by means of comparing accumulated polyphenol small intestinal cross-over. The permeability assay was performed at 37°C, over 24 h, using the flow-through diffusion system [16], where after fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) individual replicate cumulative peak area of apigenin-*O*-rutinoside; B) average replicate cumulative peak area of apigenin-*O*-rutinoside; C) individual replicate cumulative peak area of scolymoside; D) average replicate cumulative peak area of scolymoside; E) individual replicate cumulative peak area of chrysoeriol-*O*-rutinoside; F) average replicate cumulative peak area of chrysoeriol-*O*-rutinoside; G) individual replicate cumulative peak area of luteolin-*O*-hexoside; H) average replicate cumulative peak area of luteolin-*O*-hexoside; I) individual replicate cumulative peak area of apigenin-6,8 di-*C*-glucoside; J) average replicate cumulative peak area of apigenin-6,8 di-*C*-glucoside. Replicates five and six where excluded in all instances.



Supplementary Figure 7 Diffusion analysis of the dihydrochalcones originating from SM6Met via porcine small intestine by means of comparing accumulated polyphenol small intestinal cross-over relative to its starting concentration. The permeability assay was performed at 37°C, over 24 h, using the flow-through diffusion system [16], where after fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) individual replicate cumulative peak area of phloretin-3',5'-di-*C*-glucoside; B) average replicate cumulative peak area of phloretin-3',5'-di-*C*-glucoside; C)) individual replicate cumulative peak area of 3-hydroxyphloretin-3',5'-di-*C*-hexoside; D) average replicate cumulative peak area of 3-hydroxyphloretin-3',5'-di-*C*-hexoside. Replicates five and six where excluded in all instances.



Supplementary Figure 8 Diffusion analysis of the benzophenones originating from SM6Met via porcine small intestine by means of comparing accumulated polyphenol small intestinal cross-over relative to its starting concentration. The permeability assay was performed at 37°C, over 24 h, using the flow-through diffusion system [16], where after fractions were analysed using LC-MS/MS to determine the presence of polyphenols originating from SM6Met. A) individual replicate cumulative peak area of iriflophenone-di-*O*,*C*-hexoside; B) average replicate cumulative peak area of iriflophenone-di-*O*,*C*-hexoside; C) individual replicate cumulative peak area of iriflophenone-3-*C*-glucoside; D) average replicate cumulative peak area of iriflophenone-3-*C*-glucoside. Replicates five and six where excluded in all instances.

Supplementary Table 2. Calculated flux and P_{app} values from quantified SM6Met polyphenols pre and post-exclusion of replicates five and six

Compound class	Polyphenol description	g/100g SM6Met	C_o	Q	Cumm peak area relative to positive control [a]		J/ Flux[b]		Papp[c]	
					[d]	[e]	[d]	[e]	[d]	[e]
Xanthone	Mangiferin	1.899	4.634	2.317	5.28	7.56	4.34	6.21	1.56	2.23
	Isomangiferin	0.645	1.574	0.787	4.52	6.47	1.26	1.80	1.33	1.91
Flavanone	Eriocitrin (eriodictyol-7-*O*-rutinoside)	0.846	2.064	1.032	4.85	6.86	1.77	2.51	1.43	2.03
	Hesperidin (hesperetin-7-*O*-rutinoside)	2.049	5.000	2.500	8.23	11.73	7.29	10.38	2.43	3.46
Flavone	Scolymoside (luteolin-7-*O*-rutinoside)	1.289	3.150	1.573	7.96	11.48	4.43	6.40	2.35	3.39
Dihydrochalcone	Phloretin-3',5'-di-*C*-glucoside	1.278	3.118	1.560	9.04	12.68	5.00	7.01	2.67	3.74
	3-Hydroxyphloretin-3',5'-di-*C*-hexoside	0.700	1.708	0.854	5.35	7.48	1.62	2.26	1.58	2.21
Benzophenone	Iriflophenone-3-*C*-glucoside	0.669	1.632	0.817	6.48	8.93	1.87	2.58	1.91	2.64

[a] refers to accumulated polyphenol peak area at 24 h relative to the polyphenol peak area in the positive control
[b] refers to flux after 24hrs with the values represented using the following units: x 10^2 ng.cm^{-2}.min^{-1}
[c] refers to P_{app} after 24hrs with the values represented using the following units: x 10^{-6} cm.s^{-1}
Calculating J for mangiferin (post exclusion): ((((0.01899g*0.122g)*1000000000)*7.56 /100)/1440 min)*1/0.196 cm^2=6.21 x 10^2 ng.cm^{-2}.min^{-1}
1.899g/100g was the concentration of mangiferin present in SM6Met
7.56 % was the accumulated peak area of mangiferin at 24 h relative to the mangiferin peak area in the positive control
0.122 g was the amount of SM6Met loaded onto each replicate
1440 min = 24 h x 60 min
A=0.196 cm^2
P_{app} = (6.21 x 10^2 ng.cm^{-2}.min^{-1}/60)/(0.01899g*0.244g*1000000000)= 2.23: x 10^{-6} cm.s^{-1}
Calculation of Q and Co was performed as described in section 4.2.6
C_o is represented in mg/mL
Q is represented as x 10^6

Chapter 5
General discussion and conclusion

Women in menopause have shown great interest in phytoestrogenic nutraceuticals as a safer alternative to conventional HRT [1-7], more specifically a nutraceutical that would effectively address menopausal symptoms, whilst, as a positive side effect, prevent breast cancer [8,9]. As phytoestrogens binds to both ERα and ERβ, with a higher binding affinity for ERβ than ERα [10-13], phytoestrogenic preparations may serve as a source of ER subtype specific ligands [14]. This is promising for the future development of a nutraceutical as ERα activation is linked to breast cancer cell proliferation, whilst ERβ activation inhibits ERα mediated cell proliferation [15]. ERβ subtype specific ligands may thus serve as possible phytoestrogenic nutraceutical candidates, as they would be useful in the prevention and treatment of breast cancer [16-18] whilst alleviating menopausal symptoms [19-21].

A possible source of such ER subtype specific compounds has recently been identified by Mfenyana *et al*. [22] as the *C. subternata* sequential methanol extract, SM6Met. SM6Met acted as the most potent estrogenic extract compared to other *Cyclopia* extracts, with activity comparable to that of commercially available oral phytoestrogenic nutraceuticals. Subsequently the specific estrogenic profile of SM6Met was evaluated by Visser *et al*. [23] and three estrogenic traits well-suited for the future development of an effective oral phytoestrogenic nutraceutical were highlighted. These desirable traits were selective ERβ activation, antagonism of ERα activity, and antagonism of E_2-induced breast cancer cell proliferation, suggesting that either a compound or compounds in SM6Met may be suitable for the treatment of menopausal symptoms, while as a positive side effect preventing the development of breast cancer [15-18].

As part of an investigation into the possibility of SM6Met serving as a candidate source in the development of an oral phytoestrogenic nutraceutical, various drug development factors should be considered. Apart from identifying the active ingredient, determining a therapeutic dose, and establishing pharmacological properties, these factors include, amongst others, screening the compounds of interest for intestinal absorption, evaluating the effect of hepatic metabolism on

compound stability, assessing compound interaction with other drugs, and calculating compound toxicity [24-27]. In addition, the compound administered should ideally have a high therapeutic index, so that the plasma level required to exert a toxic effect would be significantly higher than that required for therapeutic efficacy [24].

The first of the two main aims of the current study was to identify the estrogenic compounds in the SM6Met extract responsible for its desired estrogenic traits. Identification of the polyphenolic compounds was approached through specific estrogenic activity-guided fractionation of the extract. Successful identification of the bioactive compound(s) would be useful not only for quality control of a future nutraceutical, but also to screen prospective *Cyclopia* harvestings destined for nutraceutical production. In addition, such a marker compound(s) could in future be used to direct plant breeding programs. The second of the two main aims of the study was to determine whether the compounds of interest can indeed permeate the intestine in order to become bioavailable. Bioavailability is a crucial factor for compounds to have a desired therapeutic effect upon oral administration, and as such, absorption of SM6Met compounds were evaluated through analysis of SM6Met compound intestinal permeability using the *ex vivo* flow-through diffusion assay.

The flow-through diffusion assay was the assay of choice for the evaluation of intestinal absorption of SM6Met polyphenols, as this approach has successfully been used in other absorption studies [28-30], and it allows for the use of intact porcine intestine. Porcine intestine has been shown to be physiologically more similar to human intestines than any other non-primate mammalian models [31]. As pharmacokinetic studies have shown that absorption may occur along the entire length of the intestine [32], our approach allowed investigation of both small and large intestinal tissue. The results from the *ex vivo* flow-through diffusion study were complemented by commonly used human absorption prediction software analysis, which are based on physicochemical profiling of the investigated compounds.

Evaluation of large intestinal permeation of SM6Met polyphenols revealed an unexpected result, which was that none of the polyphenols could penetrate this barrier. The reason for this result was attributed to the absence of colonic microflora in the investigated absorption model. Colonic microflora are responsible for degradation of polyphenols to smaller polyphenolic acids, and as a result these compounds are then more readily absorbed [33]. Small intestinal permeation, however, did occur for all compounds, except for luteolin and quercetin rutinoside/neohesperidoside, and it was found that permeation of compounds occured in a concentration-dependent manner, which is commonly seen in absorption studies [34-36]. SM6Met compound classes allowed categorisation of preferential small intestinal permeation. It was found that when considering the compound concentration in the starting mixture and comparing it to the concentration thereof in collected fractions, on average the compound classes in order from high to low permeation preference were: dihydrochalcones, benzophenones, xanthones, flavanones, and flavones.

It has been shown that *C*-glycosides are usually absorbed to a lesser extent than *O*-glycosides, as colonic microflora are less efficient in hydrolysing the more stable *C*-*C* bonds [37], however, the current study indicated that the compound classes with the highest degree of permeation were the classes containing *C*-glycosides, the dihydrochalcones, benzophenones and the xanthones. In addition, compounds containing a rutinoside moiety have been suggested to generally exhibit a lower degree of permeation compared to similar compounds containing a glucoside moiety [38], which was not found in the current study.

In an attempt to predict human intestinal absorption of SM6Met polyphenols, both experimental and computational data were used. P_{app} values were calculated from experimental data, and software programs OSIRIS [39], ACD/Labs and ChemAxon [40], based on the physicochemical profiling of compounds, were utilised, to predict absorption. P_{app} values of quantifiable compounds ranged from 1.91-3.74 x 10^{-6} cm.s^{-1} correlating with good absorption in the human body [41,42]. In contrast, the ACD/Labs program predicted more than two Lipinski “Rule-of-5” violations for all polyphenols

investigated, except for luteolin, suggesting that these polyphenols would be poorly absorbed in the human body. The OSIRIS and ChemAxon programs, on the other hand, predicted good absorption of all compounds based on their individual cLogP values (< 5) and solubility (> -4). In addition, the OSIRIS program, considering most commercially available drugs as reference, calculated a low to moderate overall drug score for the respective compounds.

Analysis of computational prediction data obtained from the various programs employed in this study revealed large variations between programs, as well as a divergence from experimental results. For future investigations experimental data should thus take priority, which could be accompanied by results obtained from a variety of commonly used, and well-established, software programs in order to obtain a more accurate prediction of the human intestinal absorption of a compound of interest. Even though the current study did not cater for the role of a number of factors that could alter the absorption of an active compound, such as the role of intestinal microflora, and compound metabolism and excretion, it did evaluate intestinal permeation, a prerequisite for orally-administered compounds to become bioavailable. If the compounds evaluated for intestinal permeation in the current study are thus re-evaluated for absorption in the future, a more holistic test-system that incorporates the role of the intestinal microflora, and compound metabolism, and excretion should be used, in an attempt to more accurately predict human absorption of these compounds. However, the variable of compound permeation has now been established in the current thesis and can therefore be incorptorated in the evaluation of results from future absorption studies.

Activity-guided fractionation combined with qHPLC was used in the isolation and identification of compounds contributing to the specific estrogenic activity of SM6Met. This approach is commonly used in the identification of compounds responsible for the specific activity of a mixture [19,43,44]. In contrast to most studies, the source of interest for the current study, SM6Met, has three attributes contributing to the holistic activity, of which two characteristics, ERα antagonism, and ERβ

agonism, are most responsible for antagonism of E_2-induced breast cancer cell-proliferation [15-18], and as such, it made for a more complex investigation. Despite the complexity of the current study, it has previously been demonstrated that one compound could display more than one estrogenic attribute. For example, R,R-THC has been shown to be both an ERα agonist and an ERβ antagonist [45-47]. However, the possibility that one compound could be responsible for both the ERα antagonism and ERβ agonism displayed by SM6Met now seems unlikely, as HPCCC fractionation of the SM6Met derived NPF and subsequent activity tests revealed that HPCCC fractions F1 and F2 acted as ERα antagonists, while HPCCC fraction F3 acted as an ERβ agonist. It is thus evident that fractionation separated the two main desired estrogenic attributes of SM6Met, which dismisses the idea that a single compound combines both attributes.

The activity-guided fractionation procedure followed in the current study included preliminary liquid-liquid fractionation of SM6Met to form two fractions, dissimilar based on their polarity, namely NPF and the PF. qHPLC analysis revealed that this procedure concentrated the compounds of SM6Met into NPF, and subsequent activity tests revealed that all of the desired estrogenic traits were consequently enhanced in NPF, but not in PF. NPF was therefore fractionated by HPCCC into fractions F1, F2, and F3. qHPLC analysis revealed that during this fractionation a differential distribution of NPF compounds into the HPCCC fractions occurred. Consequently the estrogenic traits were also separated as mentioned above, where F1 and F2 acted as ERα antagonists, and F3 acted as the ERβ agonist. In addition, only F2 antagonised E_2-induced breast cancer cell-proliferation. This process not only revealed that more than one compound is most probably responsible for the estrogenic attributes of interest in SM6Met, but also that future studies should focus on NPF as the reference source of these traits, as all the traits of interest are present in this crude fraction. Furthermore, assessment of the synergistic, additive or antagonistic effects of the mixture of compounds present in NPF, not exhibited by the polyphenols individually, could be productive.

Comparing the activity of the HPCCC fractions to literature data suggests that the only compound likely accountable for the ERα antagonism observed for HPCCC fractions F1 and F2, is eriocitrin, as this compound has been shown to bind ERα [48], and is present as a major compound in HPCCC fractions F1 and F2, but not in F3 (Fig. 6C & Table 4). However, the fact that F2 contains ten times more eriocitrin than F1, and results in the same degree of activity, suggests that, either the eriocitrin is not the only candidate responsible for ERα antagonism, or, that the degree of antagonism induced by the lower amount of eriocitrin cannot be exceeded. Interestingly, even though our own results suggested that the iriflophenone-3-*C*-glucoside-enriched (Fig. S4) fraction or the suite of minor compounds accompanying iriflophenone-3-*C*-glucoside in the enriched fraction (Fig. S4), could act as ERα and ERβ antagonists (Figs 8C&D), other studies have shown that iriflophenone-3-*C*-glucoside induces proliferation of breast cancer cells [49,50].

In the current study it was shown that the most likely candidate responsible for the robust ERβ agonism of HPCCC fraction F3 is scolymoside, as the scolymoside-enriched fraction (Fig. S3) was solely responsible for the ERβ agonist activity of HPCCC fraction F3 (Fig. 8B). However, one cannot exclude the fact that the minor compounds present in the scolymoside-enriched fraction, could also have had an influence on ERβ agonist activity, and as such future studies should investigate pure scolymoside in order to obtain more conclusive results, as this compound has recently become commercially available. Interestingly, the aglycone of scolymoside, luteolin, which is a known phytoestrogen [51], has previously been shown to antagonise E_2-induced breast cancer cell proliferation [51-53]. In contrast, our study did not show antagonism of E_2-induced breast cancer cell proliferation (Fig. 9B) in the presence of luteolin, at the concentration present in HPCCC fraction F3. This result is in accordance with the antagonism of both ERα and ERβ induced by luteolin established in the current study (Figs. 8B&D).

In a study by Li *et al*. [54] it was shown that the xanthone, mangiferin, has anti-tumour properties against MCF-7 breast cancer cells not mediated via the ER, but rather by regulating matrix

metalloproteinases, the epithelial to mesenchymal transition, and the β-catenin signalling pathway. E_2-induced breast cancer proliferation antagonism was, however, not exhibited by mangiferin in the current study at the concentration of mangiferin in HPCCC fraction F3 (Fig. 9B), despite the fact that our results indicated that mangiferin acts as an ERα antagonist, but not an ERβ antagonist (Figs. 8C&D). Interestingly, a novel result suggested that the regio-isomer of mangiferin, isomangiferin, acted as an ERβ antagonist. Another interesting result observed was that both investigated phenolic acids, *p*-coumaric acid and protocatechuic acid, displayed ERβ antagonism, while in addition, protocatechuic acid displayed ERα antagonism (Fig. 8C) and antagonism of E_2-induced breast cancer cell proliferation (Fig. 9B).

In conclusion, activity-guided fractionation of SM6Met suggests that the desired estrogenic traits of the extract cannot be explained by only one compound. Compounds present in SM6Met or NPF might thus display additive, antagonistic, and/or synergistic activities in conferring these estrogenic traits. Previous studies have shown that iriflophenone-3-*C*-glucoside, present in HPCCC fractions F2 and F3, may exert synergistic effects on other compounds present in SM6Met and NPF [55,56]. These compounds include mangiferin and hesperidin, which has been linked to anti-diabetic and cholesterol-lowering effects seen in rat models [57]. Investigation of the synergistic estrogenic effects of iriflophenone-3-*C*-glucoside and hesperidin could be interesting as both compounds are present in NPF, the iriflophenone-3-*C*-glucoside enriched fraction (Fig. S4), and in HPCCC fraction F2. Also, interestingly, it has recently been demonstrated by Bartoszewski *et al.* [58] that even though hesperidin has been shown to elicit cell growth arrest and apoptosis in various cancer cell lines including pancreatic cancer cells, colon cells, and breast cancer cells [59-62], the presence of mangiferin results in an additive apoptotic effect compared to the effect seen by hesperidin alone.

A shortcoming of the current study is that reconstitution of HPCCC fractions only included the major quantifiable compounds, and not the minor compounds, and as such, future work could include the reconstitution of HPCCC fractions F1-F3 for comparison with the original fractions

using not only pure quantifiable compounds for reconstitution, but also pure minor compounds. This will allow for a more specific and definite correlation of compounds and their related activities. Furthermore, the estrogenic assays used in the current study could be expanded to allow for a more stringent activity-guidance procedure. *In vitro* assays that could for example be included are, whole cell binding, determination of ERα and ERβ levels, ERα and ERβ hetero- and homo-dimerization, and microarray analyses to evaluate effects on endogenous genes. *In vivo* absorption analysis of the less complex HPCCC fractions F1-F3, for comparison with the current *in vitro* absorption study, and also blood samples from a previously performed *in vivo* immature rat uterotrophic study investigating SM6Met [23] (Appendix 1), can also be investigated.

The idea of intelligent mixtures in the investigation of activity, induced by a mixture of compounds, has become increasingly popular [63-68], and as NPF more prominently displays all the desired estrogenic traits of SM6Met, future work will focus on the identification and production of an intelligent mixture where NPF will serve as a reference extract. Activity analysis of intelligent mixtures would allow the investigator to establish whether synergistic, additive or antagonistic effects contribute to the desired estrogenic traits. Furthermore, it has been shown that absorption of a compound can either be increased or decreased in the presence of other compounds. For example, it has been shown that a polyphenol-based matrix, due to the presence of specific polyphenols, can result in an increase [69,70], decrease [71,72] or have no effect, on the absorption of other polyphenols [73]. In addition, polyphenols can increase drug absorption [74] through the inhibition of CYP3A4 [75,76] or the inhibition of monocarboxylate transporter 1 (MCT1) activity [77]. Absorption analysis of intelligent mixtures both *in vivo* and *in vitro*, and comparison of these results to the results obtained with the individual polyphenols, and with NPF, would reveal permeation accelarating or permeation retarding effects present in NPF, and also the compounds responsible for these effects.

In summary, the current study illustrated that it is unlikely that one compound is responsible for the desired estrogenic attributes displayed by SM6Met, but rather that multiple polyphenols contribute to the activity of the extract, either individually, or by eliciting an additive, synergistic or antagonistic effect in the presence of other SM6Met polyphenols. Furthermore, the current study established that most of the polyphenols in SM6Met could penetrate the human small intestine, and as such, these compounds could become bioavailable in order to elicit biological effects. By attempting to identify the compounds conferring phytoestrogenic activity to SM6Met, and to predict whether these compounds are able to penetrate the human intestinal barrier, valuable information was gained. Thus the current study could serve as a stepping stone in the possible future development of a uniquely South African honeybush-derived oral phytoestrogenic nutraceutical.

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Appendix 1

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Cyclopia Extracts Act as ERα Antagonists and ERβ Agonists, *In Vitro* and *In Vivo*

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Abstract

Hormone replacement therapy associated risks, and the concomitant reluctance of usage, has instigated the search for new generations of estrogen analogues that would maintain estrogen benefits without associated risks. Furthermore, if these analogues display chemo-preventative properties in breast and endometrial tissues it would be of great value. Both the selective estrogen receptor modulators as well as the selective estrogen receptor subtype modulators have been proposed as estrogen analogues with improved risk profiles. Phytoestrogen containing extracts of *Cyclopia*, an indigenous South African fynbos plant used to prepare Honeybush tea may serve as a source of new estrogen analogues. In this study three extracts, P104, SM6Met, and cup-of-tea, from two species of *Cyclopia*, *C. genistoides* and *C. subternata*, were evaluated for ER subtype specific agonism and antagonism both in transactivation and transrepression. For transactivation, the *Cyclopia* extracts displayed ERα antagonism and ERβ agonism when ER subtypes were expressed separately, however, when co-expressed only agonism was uniformly observed. In contrast, for transrepression, this uniform behavior was lost, with some extracts (P104) displaying uniform agonism, while others (SM6Met) displayed antagonism when subtypes were expressed separately and agonism when co-expressed. In addition, breast cancer cell proliferation assays indicate that extracts antagonize cell proliferation in the presence of estrogen at lower concentrations than that required for proliferation. Furthermore, lack of uterine growth and delayed vaginal opening in an immature rat uterotrophic model validates the ERα antagonism of extracts observed *in vitro* and supports the potential of the *Cyclopia* extracts as a source of estrogen analogues with a reduced risk profile.

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Introduction

Hormone replacement therapy (HRT), estrogens alone or in combination with progestins, is traditionally prescribed to women undergoing menopausal transition to alleviate symptoms associated with menopause [1], such as hot flashes, night sweats, sleeping problems, vaginal dryness, and osteoporosis [2-4]. However, a number of side effects have been associated with the use of HRT, for example, an increased occurrence of breast cancer [5,6], vaginal bleeding [7], and heart disease or strokes [6,8]. These side effects have led to reluctance among concerned consumers to use HRT and instigated a search for new estrogen analogues with an improved risk profile. Furthermore, it would be of great value if these analogues should also display chemo-preventative properties in breast tissue [9,10].

Estrogens elicit their biological effects by binding to transcription factors called estrogen receptors (ERs) in the target organ/tissue (uterus, ovary, vagina, liver, bone, and breast) [11-13]. The ER exists as two subtypes, namely ERα and ERβ [14]. Current estrogens in HRT activate both subtypes of ER in all tissues [14-19]. This attribute is beneficial in bone [18,20,21] and for hot flashes [18,21], but detrimental in the breast [6,21,22] and uterus [21,23] as it increases the risk of tumorigenesis. In contrast, the selective estrogen receptor modulators (SERMs), although not ER subtype specific [24,25], act as agonists in certain tissues, such as bone [26-28], and as antagonists in others, such as breast [9,10,29]. Although, the well-known SERMs, raloxifene and tamoxifen [30], have been shown to decrease the risk of breast cancer [18,31,32] and increase bone mineral density [26-28,33], they have also been linked to an increased risk of venous thromboembolism and

occurrence of hot flashes, and can stimulate endometrial growth [28,34-36]. SERMs are thus not considered as suitable alternatives for HRT.

Physiologically, while ERα is associated with the promotion of cell proliferation that contributes to the occurrence of breast and endometrial cancer, several studies have shown that ERβ inhibits ERα-dependent cell proliferation and could prevent cancer development [15,22,37-43]. 17β-estradiol (E_2) has similar binding affinities for the two ER subtypes [44], and the subtypes stimulate the transcription of both common and distinct subsets of E_2 target genes [13,17,39,45]. However, in many cases the degree of activation via ERβ is lower [44], despite the high ligand independent transcriptional activity of this subtype [46,47]. In light of the above, it has been suggested that the development of ER subtype specific ligands may herald the arrival of a new generation of estrogen analogues that may present a novel treatment for post-menopausal symptoms, which in addition, may prevent or decrease the occurrence of breast cancer [44,48,49]. An ideal or "designer" estrogen analogue or selective estrogen receptor subtype modulator (SERSM) has been postulated that would have the following attributes: act as an ERα selective antagonist [50], down-regulate ERα protein levels [50,51], selectively activate ERβ transcriptional pathways [15,19,24,43], and display anti-inflammatory properties by inhibiting transcription of pro-inflammatory genes to prevent the occurrence of post-menopausal osteoporosis [15,52]. Current examples of subtype specific ligands are, methyl-piperidino-pyrazole (MPP) (ERα antagonist) [53,54], diarylpropionitrile (DPN) (ERβ agonist) [55], ERB-041 (ERβ agonist) [56,57], liquiritigenin (ERβ agonist) [19], isolated from the plant extract MF101 (ERβ agonist) [24]. Phytoestrogens have been referred to as natural SERMs and can be both estrogenic as well as antiestrogenic [58-60]. Furthermore, although evidence in the literature shows that phytoestrogens can bind to both ER subtypes, they generally have a higher affinity for the ERβ subtype [61-63] as well as a higher transcriptional potency and efficacy via ERβ [63]. Despite conflicting evidence regarding doses of phytoestrogens and breast cancer risk [64,65], generally, findings have pointed the search in the direction of phytoestrogens and focused attention on phytoestrogen rich food sources as a possible source of the ideal SERSM.

One such source may be *Cyclopia* (family: Fabaceae), an indigenous fynbos plant from the Western Cape province of South Africa [66,67]. Traditionally, the "fermented" (oxidized) form of *Cyclopia*, has been consumed as a fragrant, caffeine free honeybush tea beverage with the "unfermented" form being introduced to the commercial market more recently [63,67,68]. Studies that investigated the chemical composition of *Cyclopia* have shown that phenolic compounds with estrogenic activity, for example luteolin, eriodictyol, naringenin, and formononetin, are present in various species of *Cyclopia* [63,68-72]. Furthermore, although dried methanol extracts (DMEs) from plant material of two species of *Cyclopia*, *C. genistoides* and *C. subternata*, have been shown to bind to the ERs and are able to transactivate an ERE-containing promoter reporter construct [62,63,68], only the extract from *C. genistoides* was investigated for ER subtype specificity and found to transactivate only through ERβ, despite binding to both subtypes [62,63]. In addition, studies by Verhoog et al. [63] and Mfenyana et al. [68] showed that although extracts of *Cyclopia* are able to induce proliferation of the ERα and ERβ positive MCF-7 BUS cells, they antagonise E_2 induced cell proliferation.

The current study was prompted by the findings of Verhoog et al. [62,63] that the *Cyclopia* extract, P104, although binding to both receptors and with a much higher affinity for ERα, was able to activate an ERE-containing promoter reporter construct only via ERβ. As the possibility of ERα antagonism by *Cyclopia* extracts had not been addressed in previous studies it appeared essential to evaluate ERα antagonism while also re-evaluating ERβ agonism. The combination of ERα antagonism and ERβ agonism may be especially relevant for the chemoprevention of breast cancer as ER antagonism serves as the basis of current chemo-preventative agents [29,31,32,73,74], while ERβ specific agonists have recently been identified as having potential for the chemoprevention of breast cancer [19,22]. In addition, this combination might be advantageous for the treatment of menopausal symptoms as an ERβ agonist has been shown to alleviate both hot flashes and the surge of inflammation related diseases during menopause [24,52], while an ERα antagonist would not result in hyperplasia of the uterus, commonly associated with ERα agonists [15,52]. Thus, in this study, we evaluate the potential of several extracts of *Cyclopia* to act as ERα antagonists and ERβ agonists and demonstrate that all extracts display ERα antagonism, while two also display ERβ agonism. In addition, all extracts antagonise E_2-induced MCF-7BUS cell proliferation, one extract displays anti-inflammatory activity, and the two tested extracts do not stimulate uterine growth. These results suggest that the *Cyclopia* extracts, which display ERα antagonism and ERβ agonism, have positive attributes that could possibly be further exploited for the development of safer drugs for the treatment or prevention of osteoporosis or pre-menopausal symptoms.

Material and Methods

Ethics statement

Animal care and experimental procedures were conducted with strict adherence to the accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. Stellenbosch University ethics committee approved this study (ethical approval reference: 11NB_LOU01).

Test Compounds

17β-Estradiol (E_2), genistein, luteolin, enterodiol, phorbol 12-myristate 13-acetate (PMA) and fulvestrant (ICI 182,780) were obtained from Sigma-Aldrich®, South Africa, and coumestrol was obtained from Fluka™ Analytical, Sigma-Aldrich®, South Africa. The *Cyclopia* extracts used for *in vitro* studies, P104 [62], SM6Met [68] and cup-of-tea [68], were previously prepared, while for *in vivo* studies new SM6Met and cup-of-tea extracts were prepared as previously described [68]. E_2,

genistein, luteolin, enterodiol, coumestrol and *Cyclopia* extract stock solutions were prepared in dimethylsulfoxide (DMSO).

High-performance liquid chromatography (HPLC) analysis of C. *subternata* extracts

The newly prepared SM6Met and cup-of-tea extracts were analyzed using HPLC. Extracts and stock solutions of standards were prepared in DMSO and aliquots frozen at -20°C until needed for analysis. For experimental analysis ascorbic acid was added to defrosted standards and extracts to a final concentration of 9.8 mg/ml. The mixtures were then filtered using Millex-HV syringe filters (Millipore) with a 0.22 µm pore size.

Analyses were performed on an Agilent 1200 HPLC consisting of an in line degasser, diode-array detection (DAD), column oven, autosampler and quaternary pump, controlled by Chemstation software (Agilent Technologies, Santa Clara, CA). The HPLC method previously described by De Beer et al. [75] was used to quantify the major phenolic compounds in *C. subternata* extracts: *A Gemini*-NX C18 (150 × 4.6 mm; 3 µm; 110 Å) column was used in conjunction with 2% acetic acid (A) and acetonitrile (B) as mobile phases. Injection volumes ranged from 10-20 µl for standards and 5-50 µl for the extracts. Separation was performed at a flow rate of 1 ml/min with the following mobile phase gradient: 0-2 min (8% B), 2-27 min (8-38% B), 27-28 min (38-50% B), 28-29 min (50% B), 29-30 min (50-8% B), 30-40 min (8% B); at a temperature of 30°C.

The dihyrochalcones, flavanones and benzophenones were quantified at 288 nm, whereas the xanthones, flavones and phenolic acids were quantified at 320 nm. A calibration curve consisting of seven points was set up for all the available standards (mangiferin (Sigma-Aldrich®, South Africa), isomangiferin (Chemos GmbH, Germany), luteolin (Extrasynthese, France), eriocitrin (Extrasynthese, France), hesperidin (Sigma-Aldrich®, South Africa), protocatechuic acid (Fluka™ Analytical, Sigma-Aldrich®, South Africa)) and also standards needed to calculate equivalent values (aspalathin (kind gift from Prof. Gelderblom, PROMEC unit, Medical Research Council, Tygerberg, South Africa), apigenin (Fluka™ Analytical, Sigma-Aldrich®, South Africa), and nothofagin (kind gift from Prof. Gelderblom, PROMEC unit, Medical Research Council, Tygerberg, South Africa)). Iriflophenone-3-*C*-*β*-glucoside and iriflophenone-di-*O*,*C*-hexoside was quantified using iriflophenone-3-*C*-glucoside isolated from *C. genistoides* (personal communication from Dr. D. de Beer). Scolymoside and vicenin-2 were expressed as luteolin and apigenin equivalents, respectively, as no authentic reference standards were available for these compounds. Also phloretin-3',5'-di-C-glucoside was expressed in terms of nothofagin (3-hydroxyphloretin-3'-*C*-glucoside) equivalents.

Cell Culture

COS-1, African green monkey kidney fibroblast cells (ATCC, United States of America), and MCF-7BUS human breast cancer cells [76] (a kind gift from A. Soto, Tufts University, Boston, Massachusetts, United States of America) were maintained in high glucose (4.5 g/L) Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich®) supplemented with 10% FCS (Highveld Biologicals, South Africa), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen™, South Africa), 2mM glutamine (Merck), 44mM sodium-bicarbonate (Gibco), 1mM sodiumpyruvate (Gibco, Invitrogen Corporation), and 0.1mM non-essential amino acids (Gibco). All cells were maintained in a humidified cell incubator, set at 97% relative humidity and 5% CO_2 at 37°C. For the cell proliferation assays (MTT assay) MCF-7BUS cells were withdrawn from 100 IU/ml penicillin and 100µg/ml streptomycin for seven days prior to use.

MTT assay

On day one MCF-7BUS cells were seeded into 96-well tissue culture plates at a concentration of 2500 cells/well and allowed 24 hours to settle. The next day cells were washed with 200 µl/well pre-warmed PBS and the medium was changed to DMEM without phenol red supplemented with 5% charcoal treated FCS (Highveld Biologicals, South Africa) and incubated for 24 hours. After incubation the cells were treated for 48 hours with increasing concentrations test compounds and *Cyclopia* extracts in the presence or absence of 10^{-9}M E_2 where after the colorimetric MTT (3-(4,5- dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay, adapted from Verhoog et al. [63] and Mfenyana et al. [68], was performed. Briefly, the MTT assay entails that 4 hours before the end of the incubation period the assay medium is changed to 150 µl DMEM without phenol red, but supplemented with 5% charcoal stripped FCS, and 50 µL of MTT (methylthiazolyldiphenyl-tetrazolium) (Sigma-Aldrich®) solution (5 mg/ml) is added to each well. Cells are then incubated for four hours at 37°C, the medium removed, and 200 µL of solubilisation solution (DMSO) added to each well. The plate is then covered with foil, shaken at room temperature for 5 min, and the absorbance read at 550 nm on a BioTek® PowerWave 340 spectrophotometer. All assays included a negative solvent control, which consisted of 0.1% (v/v) DMSO only. Results are expressed as fold induction relative to solvent.

Promoter reporter studies

MCF-7BUS and COS-1 cells were seeded in sterile 10 cm tissue culture plates at a concentration of 2 x 10^6 cells/plate and allowed 24 hours to settle. On day two the cells were rinsed once with sterile phosphate buffered saline (PBS) (pre-warmed to 37°C), medium changed to DMEM without phenol red supplemented with 10% charcoal treated FCS and 1% penicillin and streptomycin mixture, and cells were transfected.

Plasmids. Human (h) ERα (pSG5-hERα [77]) and ERβ (pSG5-hERβ [78]) expression plasmids were kind gifts from F. Gannon (European Molecular Biology Laboratory, Heidelberg, Germany). The ERE-containing promoter reporter construct (ERE.vit2.luc) was a kind gift from K. Korach, National Institute of Environmental Health Science, U.S. [79] and the NFκB-containing promoter reporter construct (p(IL6κB)350hu.IL6Pluc + [80]) was a kind gift from G. Haegeman, University of Ghent, Ghent, Belgium. pGL2-Basic (Promega Corporation, Madison, Wisconsin, U.S.A.) was used as an empty vector.

Transactivation. To test transactivation through ERα COS-1 cells were transfected with 150 ng hERα and 6000 ng

of an ERE-containing promoter reporter construct. To test transactivation through ERβ COS-1 cells were transfected with 150 ng hERβ, 3000 ng of an ERE-containing promoter reporter construct, and 3000 ng empty vector. MCF-7 BUS cells (which contain endogenous hERα and hERβ) were transfected with 3000 ng of an ERE-containing promoter reporter construct and 3000 ng empty vector. The amount of promoter reporter construct for each test model that was selected was determined by the highest E_2 induction achieved (Figure S1).

Transrepression. To test transrepression through ERα COS-1 cells were transfected with 150 ng hERα, 1500 ng of an NFκB-containing promoter reporter construct and 4500 ng empty vector. To test transrepression through ERβ COS-1 cells were transfected with 150 ng hERβ, 4500 ng of an NFκB-containing promoter reporter construct and 1500 ng empty vector. MCF-7BUS cells (which contain endogenous hERα and hERβ) were transfected with 6000 ng of an NFκB-containing promoter reporter construct. The amount of promoter reporter construct for each test model that was selected was determined by the most effective E_2 repression of PMA induction achieved (Figure S2)

All transfections were performed using FuGENE™ 6 transfection reagent (Roche Applied Science, South Africa) as described by the manufacturer. Cells were left for 24 hours, replated in sterile 24-well tissue culture plates at a concentration of 5 x 10^4 cells/well and allowed 24 hours to settle. Cells were treated for 24 hours with test compounds and *Cyclopia* extracts and lysed overnight with 50 µl lysis buffer [0.2% (vol/vol) Triton, 10% (vol/vol) glycerol, 2.8% (vol/vol) Tris-phosphate-EDTA, and 1.44 mM EDTA] per well at -20 °C. Luciferase activity was determined using the luciferase assay kit (Promega Corporation, Anatech, South Africa) according to the manufacturer's instructions and normalized for protein content (Bradford assay [81]). Results are expressed as fold induction relative to solvent.

Western Blot

Cell lysates from COS-1 cells transfected with either ERα (150 ng hERα/10 cm plate) or ERβ (150 ng hERβ/10 cm plate) and MCF-7BUS cells were prepared by adding lysis buffer A (10mM Hepes pH 7.5 (Gibco, Invitrogen Corporation), 1.5mM $MgCl_2$, 10mM KCl, 0.1% NP-40 (Roche Applied Science) and Complete Mini protease inhibitor cocktail (Roche Applied Science), shaking on ice for 15 min and frozen overnight at -20°C.

On thawing, lysate were transferred to 1.5ml Eppendorf tubes on ice, centrifuged for 10 min at 12 000 x g at 4°C and the cleared lysate was transferred to 1.5ml Eppendorf tubes on ice, aliquoted and stored at -20°C until assayed. Lysates (20µl) were separated on a 10% SDS-PAGE gel. Following electrophoresis, proteins were electro-blotted and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, South Africa), which was probed for ERα (diluted 1:500), ERβ (1:250) and GAPDH (1:500). Proteins were visualized using HRP labeled anti-rabbit antibody for ERα (1:2500) and ERβ (1:1000), or HRP labeled anti-mouse antibody for GAPDH (1:5000), and ECL Western blotting detection reagents (Pierce®, Thermo Fisher Scientific Inc., U.S.A.) and medical x-ray film (Axim (PTY) LTD., South Africa). All antibodies, primary [ERα (HC-20), cat# sc-543, ERβ (H-150), cat# sc-8974, and GAPDH (0411), cat# sc-47724] and secondary (anti-rabbit, cat# sc-2005, and anti-mouse, cat# sc-2030), were purchased from Santa Cruz Biotechnology, Inc., U.S.A.

Animal care

Immature female Wistar rats were obtained from the Stellenbosch University, South Africa, breeding unit and were received as weanlings on postnatal day 18. The animals had free access to standard rat feed (Pure Harvest Rat Feed, Afresh Vention (PTY) Ltd, South Africa) and drinking water. The animals were housed in a 12 hour light-dark cycle at a constant temperature of 20 °C in EHRET individually ventilated cages (EHRET, Emmedingen, Germany). The animals were allowed at least 24 hours to acclimatize before the onset of experimental procedures.

Immature rat uterotrophic assay

The immature rat uterotrophic assay was performed according to methods previously described by Kanno et al. [82] and de Lima et al. [83]. Immature Wistar rats (21 days) were randomly assigned to groups (n=10) and treated daily with E_2, genistein, *Cyclopia* extracts, or vehicle control (sterile PBS) by oral gavage for three consecutive days. The dose volume was 200 µl/day. The test compounds were suspended in sterile PBS and the solution was kept homogenous by stirring before administration. General health, vaginal opening, and body weight was monitored daily and recorded. On day four, approximately 24 hours after last dose, animals were weighed and sacrificed by administration of a high dose of Isoflurane (2-chloro-2-(difluoromethoxy)-1,1 1-trifluoro-ethane), (Safeline pharamceuticals Pty (Ltd)). Livers were removed and weighed. Uteri were removed, cleaned of excess fat, photographed, weighed, pierced to remove luminal fluids, and blotted uterine weights were obtained immediately.

Evaluation/Monitoring of vaginal opening of Wistar rats for extended period

Immature Wistar rats (21 days) were randomly assigned to groups (n=10) and treated daily with E_2, *Cyclopia* extracts, or vehicle control (sterile PBS) by oral gavage for 30 consecutive days. The dose volume had to be increased gradually from 200 µl/day to 400 µl/day as animals increased in body weight. The test compounds were suspended in sterile PBS and the solution was kept homogenous by stirring before administration. General health, vaginal opening, and body weight was monitored daily and recorded. On day 30 animals were weighed and sacrificed by administration of a high dose of Isoflurane.

Data manipulation and statistical analysis

The GraphPad Prism® version 5.10 for Windows (GraphPad Software) was used for graphical representations and statistical analysis. One-way ANOVA and Dunnett's post-test comparing all columns to the solvent control were used for statistical

analysis and significance is displayed on the graphs. For all experiments the error bars represent the SEM of at least two independent experimental repeats, except for *in vivo* studies where the error bars represent the SEM of the number of animals used.

Results

HPLC analyses of extracts of *Cyclopia*

New SM6Met and cup-of-tea extracts were prepared from the same harvesting of *C. subternata* previously used to prepare these extracts [68]. HPLC analysis was performed on these newly prepared SM6Met and cup-of-tea extracts (Table 1). Prior HPLC results of previously prepared P104 [63] and SM6Met [68] extracts are also shown in Table 1. The results indicate the presence of the xanthones, mangiferin and isomangiferin, the flavones, scolymoside, luteolin, and vicenin-2, the flavanones, eriocitin and hesperidin, the dihydrochalcones, phloretin-3,5-di-C-glucoside and aspalathin, the benzophenones, iriflophenone-3-*C*-β-glucoside and iriflophenone-di-*O*,*C*-hexoside, and the phenolic carboxylic acid, protocatechuic acid. P104, a DME from *C. genistoides*, contained more mangiferin and isomangiferin than SM6Met, a DME from *C. subternata*, while, the cup-of-tea extract from the same species contained the least. Luteolin was present in all of the extracts, albeit at small amounts, with the P104 extract containing the largest amount, followed by the SM6Met extracts, and with the cup-of-tea extract containing the least. The luteolin rutinoside, scolymoside, was not evaluated in P104. The DMEs of *C. subternata* contained more scolymoside, eriocitrin, hesperidin, and phloretin-3,5-di-C-glucoside than the cup-of-tea extract. The newly prepared DME, SM6Met, contained higher amounts than the cup-of-tea extract of compounds not previously tested for, namely, iriflophenone-3-*C*-β-glucoside, iriflophenone-di-*O*,*C*-hexoside, aspalathin, vicenin-2, and protocatechuic acid. In general the DMEs contained higher concentrations of the polyphenols quantified (Table 1) than the water extract.

Table 1. Major polyphenols present in previously and newly prepared extracts of *Cyclopia* as determined by HPLC.

	Extract			
	Previously prepared		Newly prepared	
Polyphenol [% of dry extract (g/100g dry extract)]	**P104 [63] *C.genistoides***	**SM6Met [68] *C.subternata***	**SM6Met *C.subternata***	**Cup-of-tea *C.subternata***
Mangiferin	3.606	1.850	1.899	1.000
Isomangiferin	5.094	0.750	0.645	0.420
Luteolin	0.096	0.040	0.040	0.018
Scolymoside (luteolin-7-*O*-rutinoside)	nt[a]	1.820[c]	1.289	0.876
Vicenin-2 (apigenin-6,8-di-C-glucoside)	nt	nt	0.089	0.065
Eriocitrin (eriodictyol-7-*O*-rutinoside)	nd[b]	1.250	0.846	0.600
Hesperidin (hesperitin-7-*O*-rutinoside)	nt	1.870	2.049	0.935
Phloretin-3,5-di-C-glucoside	nt	1.270[d]	1.278	0.939
Aspalathin (3-hydroxyphloretin-3', 5'-di-*C*-hexoside)	nt	nt	0.700	0.582
Iriflophenone-3-*C*-β-glucoside	nt	nt	0.669	0.590
Iriflophenone-di-*O*,*C*-hexoside	nt	nt	0.958	0.896
Protocatechuic acid	nt	nt	0.113	0.082

[a]Not tested

[b]Not detected

[c]Previously "Unknown 1" was quantified as luteolin equivalents as it appeared to be a flavone.

[d]Previously "Unknown 2" was quantified as hesperidin equivalents as it appeared to be a flavanone.

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Methanol extracts of *Cyclopia* act as agonists of ERβ, while all extracts antagonize E_2-induced activation via ERα

To evaluate ERα antagonism while also re-evaluating ERβ agonism COS-1 cells were transiently transfected with either ERα (Figures 1 A, C) or ERβ (Figures 1 B, D) and an ERE-containing promoter reporter construct. Agonism was tested in the absence (Figures 1 A, B) and antagonism in the presence (Figures 1 C, D) of 10^{-9} M E_2. Three *Cyclopia* extracts, from two species, *C. genistoides* and *C. subternata*, were tested. Two were methanol extracts, P104 and SM6Met, and one was a water extract, cup-of-tea. In addition we investigated an example from each of the major classes of phytoestrogens: genistein, a well-studied isoflavone, enterodiol, a lignin, and coumestrol, a coumestan [84,85]. Luteolin, an estrogenic polyphenol [71], was also included as it was found be present in all of the *Cyclopia* extracts (Table 1), while E_2 represents the major endogenous estrogen [86,87].

E_2 induced ERα mediated transactivation in a dose dependent manner with significant induction at two concentrations of E_2, 10^{-9} M (2.7×10^{-4} µg/ml) (2.5 ± 0.5 fold) and 9.8 µg/ml (3.6×10^{-5} M) (3.9 ± 0.7 fold), but not at the lowest concentration of 10^{-11} M (2.7×10^{-6} µg/ml) (Figure 1A). The same trend was seen for ERβ (2.5 ± 0.5 fold at 10^{-9} M and 2.7 ± 0.4 fold at 9.8µg/ml) (Figure 1B), although at the highest concentration of E_2 higher induction was observed via ERα than via ERβ (3.9 ± 0.7 vs. 2.7 ± 0.4 fold). Although the 9.8 µg/ml E_2 represents a supra-physiological concentration the 10^{-11} M and 10^{-9} M E_2 concentrations reflect the pre- and post-menopausal levels of E_2 respectively [88]. At the concentration of 9.8 µg/ml, genistein (3.6×10^{-5} M), luteolin (3.4×10^{-5} M), and coumestrol (3.7×10^{-5} M) significantly activated gene transcription through both of the ER subtypes (Figures 1A, B).

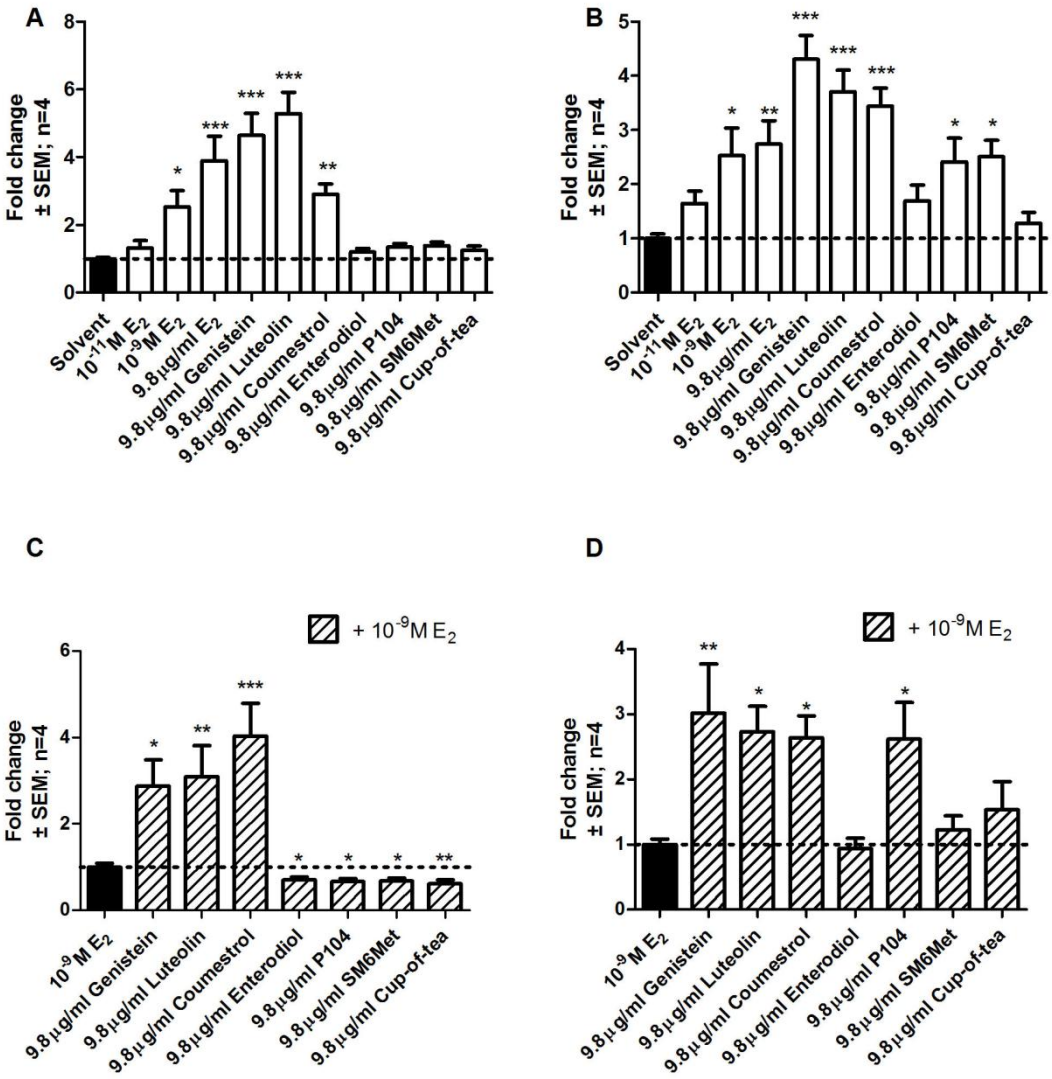

Figure 1. Evaluation of ER subtype specific agonism and antagonism of transactivation of an ERE-containing promoter reporter construct in COS-1 cells. COS-1 cells were transfected with either (A and C) pSG5-hERα or (B and D) pSG5-hERβ and ERE.vit2.luc and treated for 24 hours with a series of test compounds or extracts. To test agonism cells were treated with test compounds or extracts alone, (A and B), while, to test for antagonism cells were treated with test compounds or extracts in the presence of 10^{-9}M E_2 (C and D). Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the solvent control (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control. Average ± SEM is of four independent experiments done in triplicate.

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Enterodiol, however, could not significantly activate gene transcription through either of the subtypes at the concentration of 9.8 µg/ml (3.2×10^{-5} M) (Figures 1A, B). None of the *Cyclopia* extracts were able to induce activation through ERα (Figure 1A), but both the methanol extracts, P104 and SM6Met, were able to significantly activate transcription through ERβ (2.4 ± 0.4 and 2.5 ± 0.3 fold, respectively).

To address antagonism, transactivation in the presence of 10^{-9} M E_2 was evaluated (Figure 1C and D). The phenolic compounds, genistein, luteolin, and coumestrol were not

antagonists but had an additive effect on E_2-induced activation via both receptor subtypes (Figures 1C and D), confirming their agonism through both subtypes (Figure 1A and B). Enterodiol in contrast, however, only displays ERα antagonism (0.7 ± 0.1 fold vs. E_2 activation set as 1) (Figure 1C). All of the *Cyclopia* extracts significantly antagonized ERα mediated E_2-induction (P104, 0.7 ± 0.1, SM6Met, 0.7 ± 0.1, and cup-of-tea, 0.6 ± 0.1 fold), however, only P104 had an additive effect on the E_2-induced activation through ERβ (Figure 1D). In conclusion, the methanol extracts of *Cyclopia* are ERβ agonists and all extracts are ERα antagonists.

In MCF-7BUS cells expressing both ER subtypes all extracts of *Cyclopia transactivate* an ERE-driven promoter reporter construct

Most tissues affected by menopause and/or implicated in HRT side effects, such as uterus, bone, and breast, contain both ER subtypes [89]. Thus, having shown that methanol extracts of *Cyclopia* are ERβ agonists and all extracts are ERα antagonists in a system where the ER subtypes were evaluated separately, we were interested in investigating the transactivation potential of *Cyclopia* extracts in a system where both subtypes are present.

MCF-7BUS cells, containing both ERα and ERβ (Figure 2A), were transfected with an ERE-containing promoter reporter construct and both agonism (Figure 2B) and antagonism (Figure 2C) were tested. Although strong transactivation was seen with E_2, none of the polyphenols on their own were able to significantly activate gene transcription in this system where both ER subtypes are present (Figure 2B), despite the fact that these polyphenols transactivate when the ER subtypes function in isolation (Figure 1A and B). Furthermore, most of the polyphenols, excluding coumestrol, antagonized E_2 induction when both ER subtypes are together (Figure 2C), whereas when the subtypes were expressed separately only enterodiol showed ERα antagonism (Figure 1C). In contrast to the polyphenols, the extracts of *Cyclopia*, P104 (3.4 ± 0.5 fold), cup-of-tea (3.4 ± 0.5 fold) and, SM6Met (3.5 ± 0.6 fold), were able to activate transcription to a similar extent as 10^{-9} M E_2 (3.8 ± 0.3 fold) (Figure 2B). These results, together with the fact that the *Cyclopia* extracts did not antagonize E_2 induction (Figure 2C), suggests that when both ER subtypes are co-expressed the *Cyclopia* extracts act as agonists, whereas when the ER subtypes are expressed separately they only act as agonists through ERβ and antagonize ERα induction.

An extract of *C. genistoides* represses NFκB activation via ERα and ERβ whereas the extracts of *C. subternata* are ERβ antagonists.

The decline in estrogen levels during menopause leads to a surge in the occurrence of inflammatory disorders [52,90-92]. Furthermore, NFκB, a pro-inflammatory transcription factor, is involved in the development of breast cancer [93-95]. Taking this into account we wanted to evaluate the ability of *Cyclopia* extracts to repress the activation of an NFκB-containing promoter reporter construct by transfecting COS-1 cells with said construct and either ERα (Figures 3A, C, E) or ERβ (Figures 3B, D, F). In addition, this system would provide information concerning the behavior of *Cyclopia* extracts in a transrepression model. Agonism was tested in the absence (Figures 3A, B) and antagonism (Figures 3C, D) in the presence of 10^{-9} M E_2.

PMA (phorbol 12-myristate 13-acetate, an activator of NFκB driven gene expression [96,97]) activation of the NFκB-containing construct was repressed by E_2 via both receptor subtypes (Figure 3A and B) with a more pronounced repression through ERα (38.6% vs. 27.2%). Like E_2, all of the polyphenols, as well as P104 (*C. genistoides* extract), acted as ERα agonists by repressing PMA activation (genistein 52.1%, luteolin 50.6%, enterodiol 57.4%, coumestrol 61.8%, and P104 59.2%) (Figure 3A). Furthermore, genistein (34.8% repression) and P104 (40.7% repression), like E_2, also displayed significant ERβ agonism (Figure 3B). Therefore, in our transrepression model P104 is not an ERβ selective agonist, but displays agonism via both subtypes. The water extract of *C. subternata*, cup-of-tea, was unable to repress PMA induction through either ERα or ERβ (Figures 3A, B) while the methanol extract, SM6Met, also unable to repress PMA induction through either subtype, significantly added to the activation observed with PMA alone via ERα (5.1 ± 0.5 vs. 3.5 ± 0.5) (Figure 3A, B).

Antagonism was evaluated in the presence of 10^{-9} M E_2 and only genistein (Figure 3C) had a significant effect via ERα by antagonizing E_2 repression of PMA activation. The polyphenols, luteolin, enterodiol, and coumestrol, but not genistein, however, antagonized E_2 repression of PMA activation via ERβ (Figure 3D). Although none of the extracts displayed significant antagonism of ERα, the extracts of *C. subternata* displayed ERβ antagonism (Figure 3D).

The result for SM6Met in Figure 3A prompted us to investigate whether this effect was via ERα or if SM6Met is able to activate the NFκB-containing construct through another mechanism of action. Therefore, we repeated the experiment, for both receptor subtypes, with SM6Met, as well as P104, in the presence and absence of an ER antagonist, ICI 182,780 (Figures 3E, F). The observed repression of PMA activation by E_2 and P104 via ERα and ERβ is abolished by ICI (Figure 3E, F) and thus, the observed repression is indeed via the ER. SM6Met, like ICI, increases PMA activation through ERα (Figure 3E) and both have no significant effect on PMA activation via ERβ (Figure 3F). Furthermore, the increased transactivation observed with SM6Met in Figure 3A may be attributed to residual E_2 remaining after stripping of FCS, as suggested by others [22], which would further support the contention that SM6Met is behaving as an ERα antagonist. In conclusion then the results suggest that for our transrepression model the methanol extract of *C. genistoides* (P104) is behaving like an ERα and ERβ agonist, while the methanol extract of *C. subternata* (SM6Met) is an ERα antagonist in the absence of E_2, and an ERβ antagonist in the presence of E_2.

In MCF-7BUS cells expressing both ER subtypes all extracts are agonists, while the water extract of *C. subternata* also displays antagonistic activity.

As we have shown that P104 is an ER agonist and SM6Met is an ER antagonist in a transrepression model where the ER subtypes function in isolation (Figure 3), we wanted to test the effect of these extracts in a model where both subtypes are

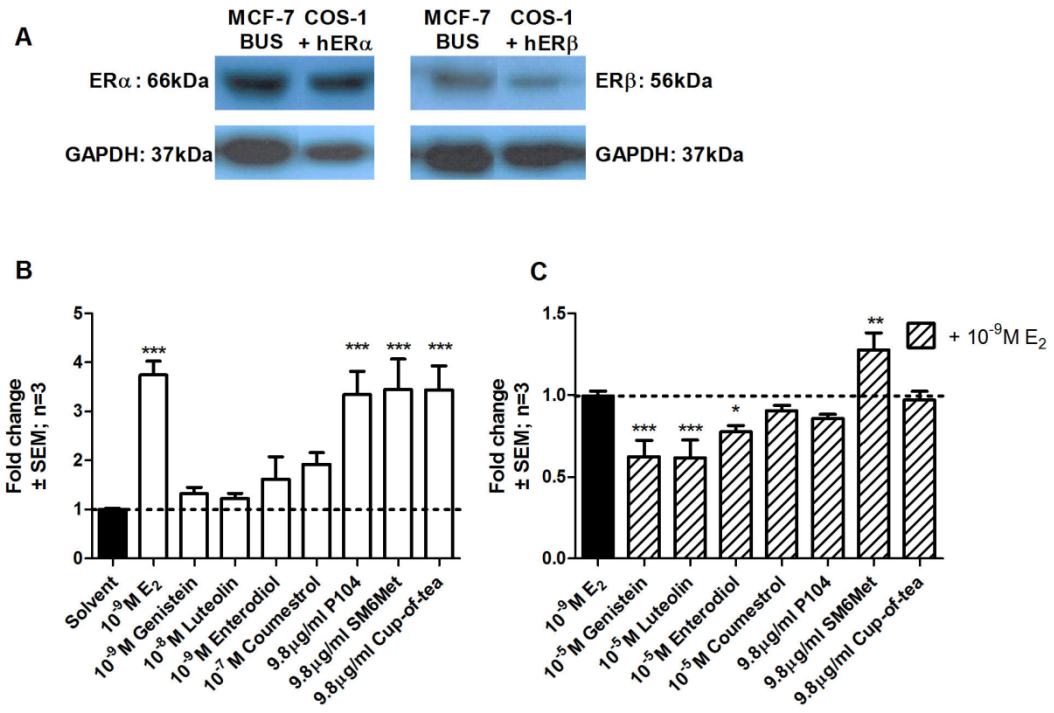

Figure 2. Evaluation of transactivation of an ERE-containing promoter reporter construct in MCF-7BUS cells expressing both ERα and ERβ. MCF-7BUS cells, with endogenous ERα and ERβ (A), were transfected with ERE.vit2.luc and treated for 24 hours with a series of test compounds or extracts. To test agonism cells were treated with test compounds or extracts alone (B), while, to test for antagonism cells were treated with test compounds or extracts in the presence of 10^{-9}M E_2 (C). Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the solvent control (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control. Average ± SEM is of three independent experiments done in triplicate.

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present as most tissues affected by menopause and/or implicated in HRT side effects contain both subtypes.

MCF-7BUS cells were transfected with an NFκB-containing promoter reporter construct and both agonism (Figure 4A) and antagonism (Figure 4 B) evaluated. Strong repression was observed with E_2, the polyphenols, and P104 when both subtypes are present (Figure 4A), which correlates with what was observed previously for ERα alone (Figure 3A). However, for ERβ alone (Figure 3B), significant repression was previously seen only with E_2, genistein, and P104 but not with luteolin, enterodiol, and coumestrol. Unlike previous results, SM6Met behaved differently when subtypes were co-expressed than when the subtypes were expressed separately. It displayed agonism when subtypes are expressed together (Figure 4A) while displaying antagonism when expressed separately (Figure 3A and D). Similarly, where no agonist activity via either subtype alone was observed previously, the cup-of-tea extract was able to change its behavior when both subtypes are present by displaying ER agonism. Furthermore, antagonism in the presence of both subtypes was only seen with the cup-of-tea extract (Figure 4B), while the subtype specific antagonism of genistein, luteolin, enterodiol, coumestrol, and SM6Met (Figures 3C, D) is abrogated in the presence of both subtypes. Taken together, in a transrepression model, the DME of *C. genistoides*, P104, is an ER agonist in all models (Figures 3A, B, and 4A), the DME of *C. subternata*, SM6Met, is an ERβ antagonist in the presence of E_2 (Figure 3D), an ERα antagonist in the absence of E_2 (Figure 3A, E), and an agonist in the presence of both ER subtypes (Figure 4A), while the water extract of *C. subternata*, cup-of-tea, is an ERβ antagonist (Figure 3D) and an ER agonist/antagonist (Figures 4A, B) in the presence of both subtypes. This differential behavior of the *Cyclopia* extracts in the transrepression model contrasts to similar behavior by the extracts in the transactivation model where all extracts displayed antagonism through ERα (Figure 1) alone, while displaying agonism to ERβ (Figure 1) alone or when both subtypes are expressed (Figure 2).

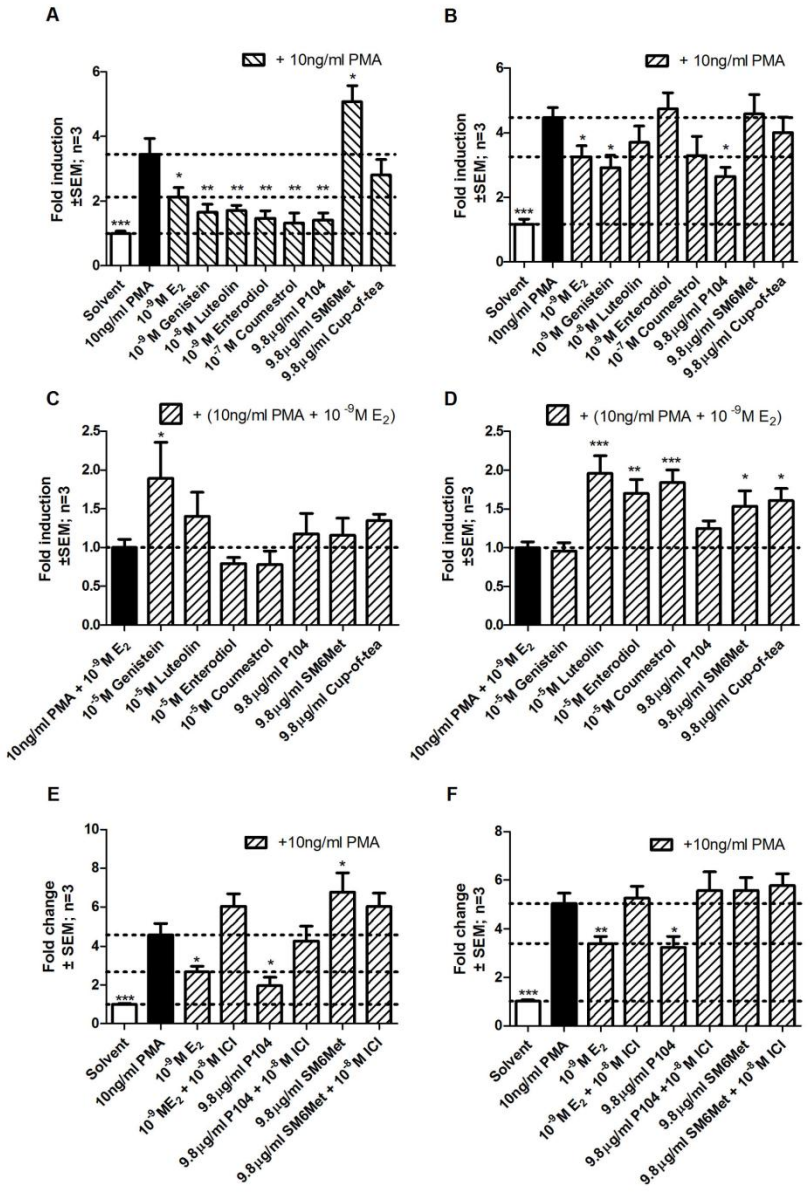

Figure 3. Evaluation of ER subtype specific agonism and antagonism of an NFκB-containing promoter reporter construct in COS-1 cells. COS-1 cells were transfected with either (A, C, and E) pSG5-hERα or (B, D, and F) pSG5-hERβ and p(IL6kB)350hu.IL6Pluc+ and treated for 24 hours with a series of test compounds or extracts. To test agonism cells were treated with test compounds or extracts alone, (A and B), while, to test antagonism cells were treated with test compounds or extracts in the presence of 10^{-9} M E_2 (C and D). To ascribe the observed effect to the ER we treated cells with P104 and SM6Met in the absence or presence of the ER antagonist ICI 182,870 (E and F). Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to either (A, B, E, and F) 10ng/ml PMA or (C and D) 10ng/ml PMA + 10^{-9}M E_2 (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted lines through the bars represent the values for either (A, B, E, and F) solvent control, 10ng/ml PMA, or 10ng/ml PMA + 10^{-9}M E_2 or (C and D) 10ng/ml PMA + 10^{-9}M E_2. Average ± SEM is of three independent experiments done in triplicate.

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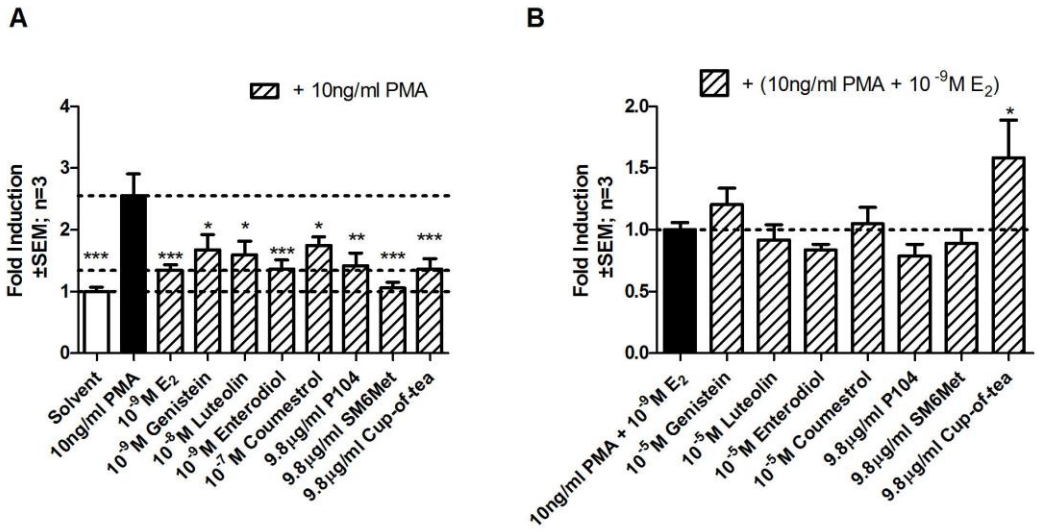

Figure 4. Evaluation of transrepression of an NFκB-containing promoter reporter construct in MCF-7BUS cells expressing both ERα and ERβ. MCF-7BUS cells were transfected with p(IL6kB)350hu.IL6Pluc+ and treated for 24 hours with a series of test compounds or extracts. To test agonism cells were treated with test compounds or extracts alone, (A), while, to test for antagonism cells were treated with test compounds or extracts in the presence of 10^{-9}M E_2 (B). Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to either (A) 10ng/ml PMA or (B) 10ng/ml PMA + 10^{-9}M E_2 (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted lines through the bars represent the values for either (A) solvent control, 10ng/ml PMA, or 10ng/ml PMA + 10^{-9}M E_2 or (B) 10ng/ml PMA + 10^{-9}M E_2. Average ± SEM is of three independent experiments done in triplicate.
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Cyclopia extracts weakly induce proliferation of breast cancer cells but antagonizes E_2-induced breast cancer cell proliferation

Having shown that *Cyclopia* extracts can modulate both transactivation and transrepression in the presence of both ER subtypes and when the subtypes are expressed alone, we wanted to re-evaluate agonism of P104 [63], SM6Met and cup-of-tea [68] (Figure 5) and antagonism of P104 [63] (Figure 6) and for the first time evaluate antagonism of SM6Met and cup-of-tea (Figure 6) on MCF-7BUS breast cancer cell proliferation. Cell proliferation in MCF-7BUS cells constitutes an integrated model where not only the ER subtypes are co-expressed, but both transactivation and transrepression of endogenous genes contribute towards the final phenotype, whether it is proliferative or anti-proliferative [39,98-100].

The MTT cell proliferation assay using MCF-7BUS cells was used to address agonism (Figure 5A-H). Estrogen induced cell proliferation at a wide range of concentrations (10^{-6} M to 10^{-10} M) with the highest efficacy (2.1 ± 0.1 fold) observed at 10^{-9} M E_2 (2.7 x 10^{-4} µg/ml) (Figure 5A). Like E_2, all of the polyphenols were also able to induce cell proliferation, but not to the same extent as E_2, with a maximum efficacy of: genistein, 1.5 ± 0.1 fold at 10^{-9} M (2.7 x 10^{-4} µg/ml) (Figure 5B), luteolin, 1.5 ± 0.1 fold at 10^{-5} M (2.7 µg/ml) (Figure 5C), coumestrol, 1.6 ± 0.1 fold at 10^{-6} M (3.0 x 10^{-1} µg/ml) (Figure 5D), and enterodiol, 1.3 ± 0.1 fold at 10^{-9} M (3.0 x 10^{-4} µg/ml) (Figure 5E). Similarly, all three extracts of *Cyclopia* induced proliferation of cells with a lower efficacy than E_2 with maximum efficacies of 1.5 ± 0.2 (significantly different from E_2), 1.3 ± 0.03 (significantly different from E_2), and 1.7 ± 0.2 (not significantly different from E_2) fold for 9.8 µg/ml of P104, cup-of-tea and SM6Met, respectively (Figures 5F-H). The potencies, depicted by EC_{50} values on graphs (Figures 5A-H), of the polyphenols, as well as of the *Cyclopia* extracts, were lower than that of E_2 with coumestrol, P104, and SM6Met significantly lower and may be listed in order of decreasing potency as follow: E_2 > genistein > enterodiol > luteolin > cup-of-tea > P104 > coumestrol >> SM6Met.

To address antagonism (Figure 6A-G), increasing concentrations of the polyphenols and *Cyclopia* extracts were tested in the presence of 10^{-9} M E_2 (highest efficacy, Figure 5A). Genistein (Figure 6A) and enterodiol (Figure 6D), significantly repressed E_2-induced cell proliferation (23.3% at 10^{-5} M (2.70 µg/ml) and 24.5% at 10^{-5} M (3.02 µg/ml), respectively). Although, luteolin (Figure 6B) and coumestrol (Figure 6C) displayed no significant antagonism, coumestrol did have a significant additive effect (1.3 ± 0.1 fold) at 10^{-9} M (2.96 x 10^{-4} µg/ml), suggesting agonism. Similarly, genistein, an antagonist at high concentrations, also had a significant additive effect (1.2 ± 0.1 fold) at the lower concentration of 10^{-8} M (2.70 x 10^{-3} µg/ml) (Figure 6A). All extracts of *Cyclopia* were able to antagonize E_2-induced cell proliferation, with P104 repressing 19.8% at 9.8 x 10^{-1} µg/ml, SM6Met 16.8% 9.8 x 10^{-4}

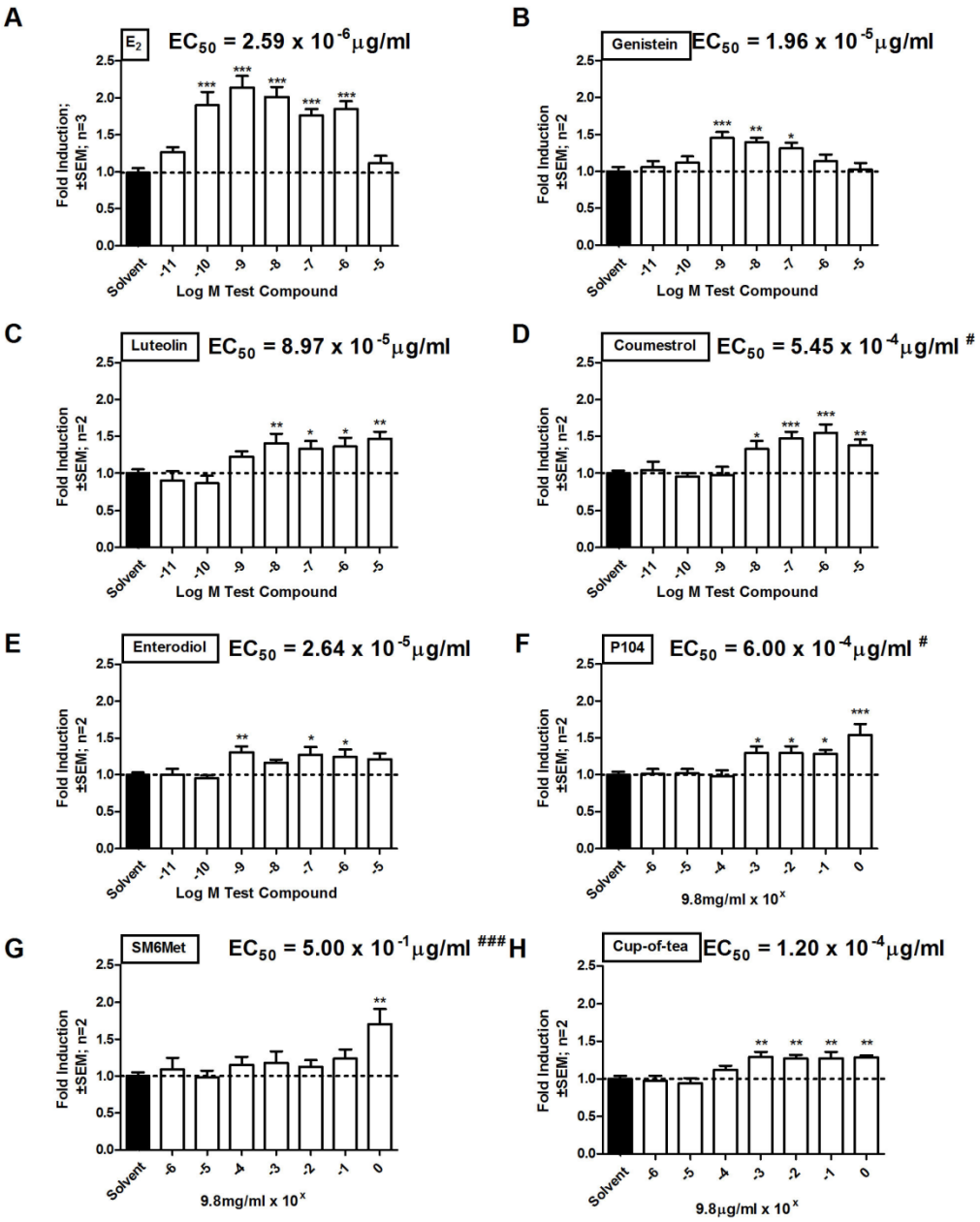

Figure 5. Evaluation of agonism of proliferation, a more complex endpoint encompassing both transactivation and transrepression in MCF-7BUS cells expressing both ERα and ERβ. MCF-7 BUS cells were treated with increasing concentrations of (A) E_2, (B-E) polyphenols, and (F-H) *Cyclopia* extracts for 48 hours. After treatment the amount of living cells was determined using a MTT assay. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the solvent control (*, P<0.05; **, P<0.01; ***, P<0.001) or to E_2 for EC_{50} values (#, P<0.05; ##, P<0.01; ###, P<0.001). The dotted line through the bars represents the values for solvent control. Average ± SEM is of two independent experiments done in six replicates, except (A) where average ±SEM is of three independent experiments done in six replicates.

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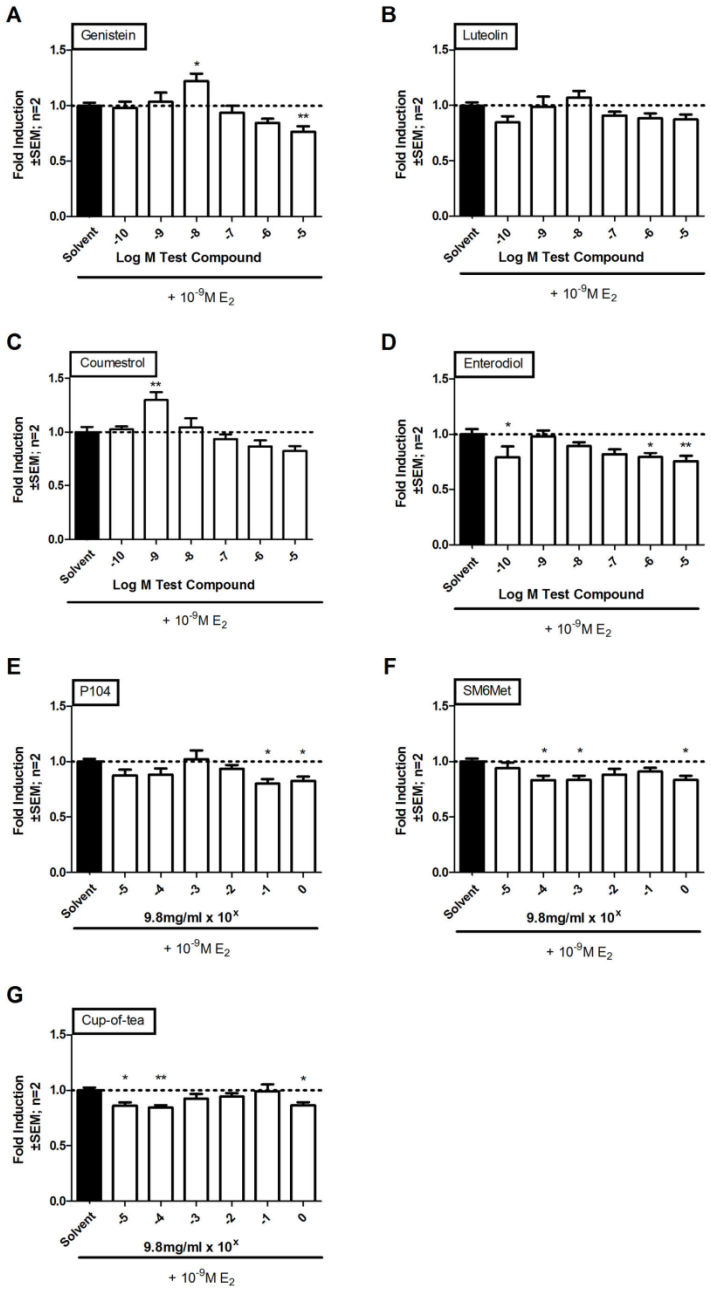

Figure 6. Evaluation of antagonism of proliferation, a more complex endpoint encompassing both transactivation and transrepression in MCF-7BUS cells expressing both ERα and ERβ. MCF-7 BUS cells were treated with increasing concentrations of (A-D) polyphenols and (E-G) *Cyclopia* extracts for 48 hours in the presence of 10^{-9}M E_2. After treatment the amount of living cells was determined using a MTT assay. Statistical analysis was done using One-way ANOVA with Dunnett's post-test comparing all columns to the solvent control (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control. Average ± SEM is of two independent experiments done in six replicates.

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μg/ml, and cup-of-tea 15.6% repression at 9.8 x 10^{-4} μg/ml (Figures 6E, F, G). Taken together, these results show that although all extracts of *Cyclopia* induced cell proliferation, the P104 and cup-of-tea extracts did so at a significantly lower efficacy and the methanol extracts at a significantly lower potency than E_2 and that all extracts could antagonize E_2-induced cell proliferation.

SM6Met does not stimulate the growth of rat uteri, antagonizes E_2-induced uterine proliferation, and delays vaginal opening

For the *in vivo* studies only extracts from *C. subternata* was used as P104 plant material was not available in bulk. The immature rat uterotrophic assay is used to determine the ability of test compounds to stimulate ERα induced uterine growth as ERβ is not highly expressed in the uterus [56,101] and also allows for the detection of antiestrogens [102]. Rats were administered E_2, genistein, and the two *C. subternata* extracts, SM6Met and cup-of-tea, via oral gavage and the effects on uterine growth were evaluated (Figure 7A, B, and Figure S3). Estrogen, as well as genistein, induced uterine growth (2.5 ± 0.2 and 2.0 ± 0.2 fold, respectively) (Figure 7). In contrast, the extracts significantly reduced uterine weight relative to solvent (Figure 7 and Figure S3). SM6Met also significantly repressed E_2-induced uterine growth by 33.0%, a result that is similar, but less pronounced, than that seen with ICI 182,780 (59.7% repression) (Figure 7) suggesting that the extracts behave as antiestrogens in the uterus.

We also addressed body weight changes and toxicity (Figure S4) and found that E_2 significantly increased body weight, whereas genistein significantly decreased body weight. The extracts of *Cyclopia* and ICI 182,780, however, did not lead to significant weight gain or loss as compared to solvent, except for the animals treated with the highest concentrations (2000mg/kg BW) of SM6Met and cup-of-tea extracts which gained significantly less weight than the solvent treated animals. With regards to toxicity, none of the treated animals showed any significant changes in liver weight, except for a decrease in liver weight in animals treated with 200mg/kg BW SM6Met.

Furthermore, as another marker of estrogenic activity, albeit a less sensitive marker [102], we also evaluated time of vaginal opening over an extended period of daily treatments (Figure 8). Estrogen led to premature vaginal opening when compared to solvent (4.2 ± 0.4 vs. 14.3 ± 1.2 days). This correlates with the observed increase in uterine weight in Figure 7. The significantly delayed vaginal opening of SM6Met treated animals (19.0 ± 1.3 days) also correlates with uterine weight results in displaying antiestrogenic behavior. The significant delay in vaginal opening was observed for all three of the concentrations of SM6Met, however, although the cup-of-tea extract showed a similar trend, it was not significant (Figure S5).

To summarize, for the first time we show that the *C. subternata* extracts are absorbed when administered orally and elicit a biological effect *in vivo*. Specifically, *Cyclopia* extracts, in contrast to E_2 and genistein, did not induce uterine growth and SM6Met antagonized E_2-induced uterine growth. Furthermore, the extracts also delayed vaginal opening in contrast to E_2. These results suggest that the *Cyclopia* extracts display ERα antagonism *in vivo* by retarding uterine growth [56,101].

Discussion

HRT in the form of estrogens provides relief from the plethora of menopause associated symptoms [1]. Although these estrogens provide relief from menopausal symptoms, they introduced new HRT associated risks, including an increased occurrence of breast cancer, heart disease, strokes, and endometrial cancer [1,5,6,8]. These risks, and the associated reluctance of usage, instigated the search for a new generation of estrogen analogues that would provide the benefits of estrogens without the associated risks. In addition, it would be of great value if these new analogues display chemo-preventative properties in breast and endometrial tissues [9,10,29].

The search for new estrogen analogues heralded the era of the SERMs. These SERMs would selectively modulate estrogen receptors in different tissues, acting as antagonists in the breast and uterus (chemo-preventative) and as agonists in the bone (osteoporosis prevention). Tamoxifen, a first generation SERM, provided the desired protective effect in the breast [31,32] and raloxifene, a second generation SERM, had protective properties in breast and bone tissues [26,27,103]. However, as these SERMS have been linked to the increased occurrence of hot flashes and stimulated endometrial growth (tamoxifen), the search continues [28,34,35]. Third generation SERMs, such as lasoxifene and bazedoxifene, are currently in development, but the focus has shifted to osteoporosis treatment with protection against breast cancer as a beneficial side effect [104-106].

Although SERM development continues there is increased interest in SERSMs, analogues that can differentially modulate specific ER subtypes. This was brought on by studies that have shown that ERβ inhibits ERα dependent cell proliferation and could prevent cancer development [15,22,37,40-43]. Phytoestrogens have been shown to be both estrogenic as well as antiestrogenic and while they can bind to both ER subtypes, they generally have a higher affinity for ERβ as well as a higher transcriptional potency and efficacy via ERβ [61-63]. Thus, phytoestrogen rich food sources have become important potential resources of SERSMS.

The current study evaluated previously described extracts of *Cyclopia*, a source of phytoestrogens, for ER agonism and/or antagonism (summarized in Table S1). Specifically, we evaluated the effect of *Cyclopia* extracts on transactivation and transrepression in a model where ERα and ERβ were expressed separately. This allows for the evaluation of the modulation of ER subtype specific activity in two transcriptional models: a classical ERE transactivation model and an NFκB transrepression model. In the transactivation model the methanol extracts, P104 and SM6Met were ERβ agonists, while all extracts antagonized ERα. In the transrepression model, however, the behavior of the *Cyclopia* extracts became more complex. P104, which displayed opposite effects via the

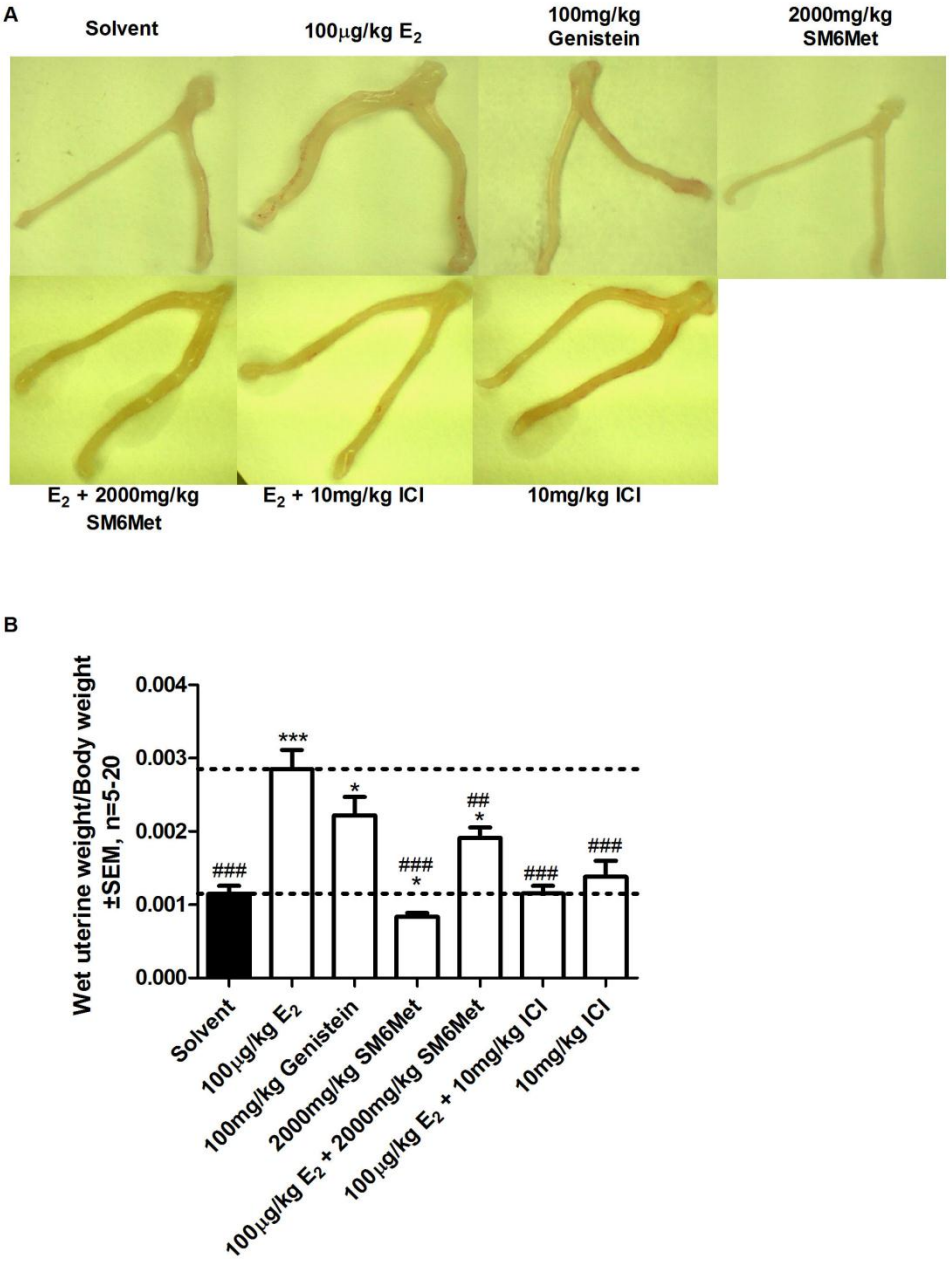

Figure 7. Evaluation of the *in vivo* effect of E_2, genistein and SM6Met on immature rat uterine growth. Immature female wistar rats were treated with 100μg/kg body weight E_2, in the presence and absence of 2000mg/kg body weight SM6Met or 10mg/kg body weight ICI 182,780, 100mg/kg body weight genistein, 2000mg/kg body weight SM6Met, and 10mg/kg body weight ICI 182,780 for three consecutive days. Animals were sacrificed on day four, (A) uteri were photographed, and (B) wet uterine/final body weight was determined. One-way ANOVA with Dunnett's post-test comparing all columns to either solvent control (*, P<0.05; **, P<0.01; ***, P<0.001) or E_2 (#, P<0.05; ##, P<0.01; ###, P<0.001). The dotted lines through the bars represent the values for solvent control or E_2. Average ± SEM is of at least five animals/group.

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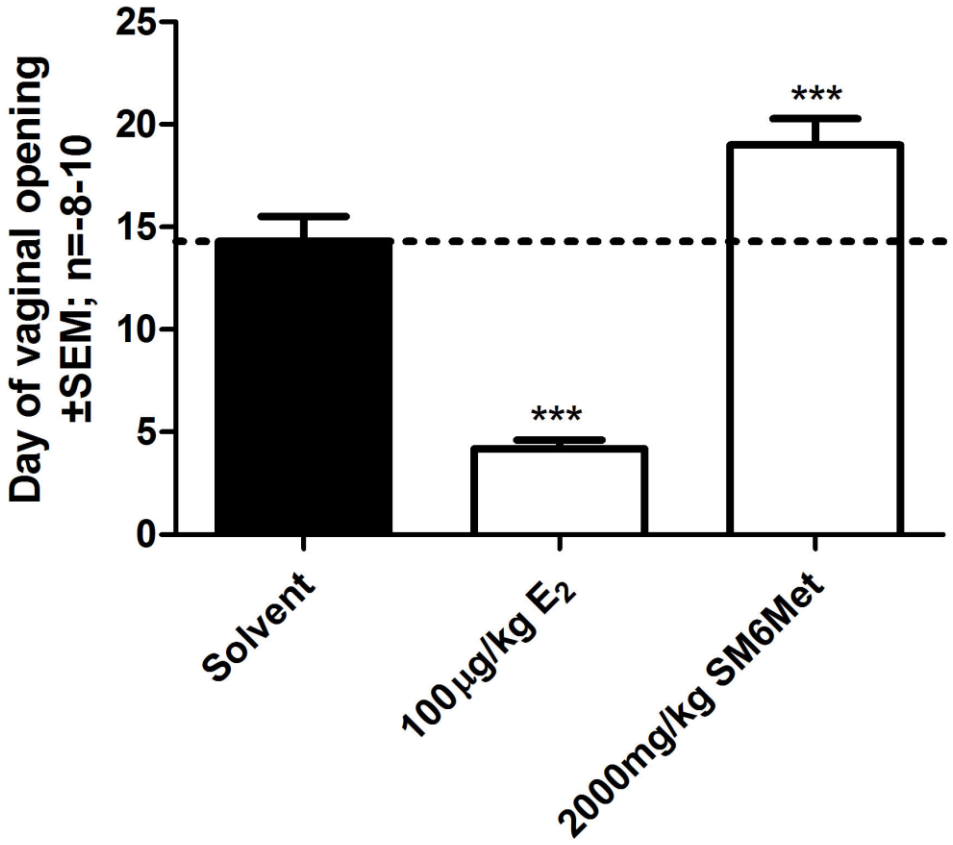

Figure 8. Evaluation of the effect of E_2 and SM6Met on the timing of vaginal opening. Immature female wistar rats were treated for 30 consecutive days with 100µg/kg body weight E_2 and 2000mg/kg body weight SM6Met and the day of vaginal opening was determined. One-way ANOVA with Dunnett's post-test comparing all columns to solvent control (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control. Average ± SEM is of at least eight animals/ group.

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subtypes in the transactivation model, acted as an agonist for both subtypes in the transrepression model. The extracts of *C. subternata*, however, did not elicit such uniform effects in the transrepression model. SM6Met, a methanol extract, acting as an ERα antagonist and ERβ agonist regarding transactivation, displayed antagonism towards ERα, in the absence of E_2, and towards ERβ, in the presence of E_2. Similar antagonism towards ERα in the absence of E_2 has also been seen for the plant extract MF101 regarding IL6 mRNA expression [24]. The water extract, cup-of-tea, also changed its behavior, acting as an ERβ antagonist for transrepression as opposed to an ERα antagonist for transactivation. These behavioral changes were not exclusive to the *Cyclopia* extracts as the polyphenols also displayed these characteristics. Luteolin, for example, displayed ER agonism through both subtypes in the transactivation model but was an ERα agonist and an ERβ antagonist in the transrepression model. The occurrence of mixed agonism and antagonism towards ER subtypes has also been observed for the xenoestrogen, Bisphenol A (BPA) [107].

As the current experiments were performed in the same cell line we have to look towards differences between the mechanisms of transactivation and transrepression for clarification of these results. Classically, transactivation is a product of ER dimer binding directly to the DNA sequence, however, tethering to DNA bound transcription factors (TFs) in the promoter region of affected genes has also been described [108-111]. Binding of the ER to DNA, whether it is direct or indirect, initiates the recruitment of co-activators, which then modulates transcription [112]. Regarding transrepression, specifically the repression of NFκB driven genes, various mechanisms of ER mediated transrepression have been described [109]. The ER can bind to NFκB and thereby prevent

DNA binding of the transcription factor [113,114], ligand bound ER present at promoter regions can recruit co-repressors [115,116], ligand bound ERα and activated NFκB can compete for co-activator recruitment [117,118], or ERα, through a non-genomic pathway, inhibits translocation of activated NFκB to the nucleus [119]. We can use this knowledge of the mechanism of action and combine it with what we know about SERMs and ER subtypes specific ligands to postulate a mechanism of action of *Cyclopia* agonism and antagonism. For the SERMs, three mechanisms of antagonism have been proposed [18]. SERMs can bind to the ER with a higher affinity than E_2 and block the binding of E_2, they can block the binding of co-activators, or SERMs can induce the recruitment of co-repressors [18,120,121].. Not much is known regarding the mechanism of SERM agonism [18], although it has been suggested that they can block the binding of co-repressors [121]. In addition, MF101 and liquiritigenin, both ERβ selective agonists, although being able to bind to ERα, cannot recruit co-activators to ERα, and MF101 cannot promote the interaction of ERα with regulatory elements [15,24]. Furthermore, it has been suggested that SERMs may activate cell surface signaling pathways that results in ligand-independent activation of ERs [29,122,123].

Therefore, with regards to transactivation, we may postulate that the extracts of *Cyclopia* cannot transactivate via ERα as they are unable to recruit the necessary co-activators, while for ERβ, P104 and SM6Met are able to do so. It is also possible that the extracts of *Cyclopia* cannot induce ERα interaction with regulatory elements. The observed ERα antagonism of E_2-induced transactivation may be due to the extracts binding to ERα and either inhibiting E_2 binding, inhibiting the recruitment of co-activators or stimulating the recruitment of co-repressors. In our transrepression model P104 behaves like E_2 and could be exerting its function by any of the NFκB repression models discussed earlier. However, SM6Met displays ERα antagonism in the absence of E_2 and this antagonism is lost in the presence of E_2. Therefore, it is possible that SM6Met is unable to recruit co-repressors in the absence of E_2 and is unable to inhibit the E_2-induced recruitment of co-repressors. Furthermore, antagonism of ERβ in the transrepression model by SM6Met and cup-of-tea may be due to the recruitment of co-activators to ERβ.

Next we evaluated agonism and antagonism of *Cyclopia* extracts in a more complex environment where the ER subtypes are co-expressed. We used the MCF-7BUS cells, a breast carcinoma cell line, not only because it co-expresses the subtypes (Figure 2A), but also to evaluate the activity of the extracts in breast tissue cells. With regards to transactivation, all extracts of *Cyclopia* were agonists and are likely exerting this agonism through ERβ as they were ERβ agonists and ERα antagonists in COS-1 cells. Also, previously we discussed the possibility that the extracts may be unable to recruit co-activators to ERα or induce ERα-regulatory element interactions, which supports the idea that the *Cyclopia* extracts are mediating their transactivative effects in MCF-7BUS cells via ERβ. Interestingly, the polyphenols, genistein and luteolin, having displayed ER agonism in COS-1 cells, in an environment where both ER subtypes are present displayed only weak agonism, which may be attributed to the fact that lower concentrations were used in MCF-7BUS cells. However, when both subtypes are present these polyphenols display antagonism, which was not apparent when the subtypes were expressed separately. When both ER subtypes are expressed in the transrepression model, all the polyphenols as well as the *Cyclopia* extracts acted as agonists, while the water extract of *C. subternata* also displayed ER antagonism. The ER agonism of P104 in the transrepression model is thus not a cell type selective effect as it is seen in both the COS-1 (kidney) and MCF-7BUS (breast) cells. The ER antagonism of cup-of-tea in MCF-7BUS cells is likely mediated via ERβ as ERβ antagonism was observed in COS-1 cells transfected with ERβ, but not in cells transfected with ERα. However, the SM6Met extract, which displayed antagonism for ERα and ERβ in COS-1 cells, changes its behavior in the MCF-7BUS cells and acts as an ER agonist in the transrepression model. Furthermore, a similar switch in behavior is observed with the polyphenols as the subtype specific antagonism is abrogated in the presence of both ER subtypes. These observed behavioral changes of the *Cyclopia* extracts as well as the polyphenols in different tissues have also been observed for the SERM, tamoxifen [18]. Ball et al. [18] found that tamoxifen differentially regulated ER regulated genes in different cell lines and ascribed this phenomenon to the presence, or lack of, co-regulators in different tissues. Therefore, the differential effect of *Cyclopia* extracts as well as the polyphenols in cells from different tissues might be due to changes in the co-regulator environment.

As MCF-7BUS cells express both ER subtypes, we also have to consider the possibility of ERα/β heterodimer formation and the biological relevance thereof as opposed to homodimer formation in COS-1 cells expressing the ER subtypes in isolation. Using two phytoestrogens that are ERα/β heterodimer selective, cosmosiin and angolensin, it was shown that heterodimer formation, in the presence of these ligands, leads to higher activation of an ERE-promoter reporter construct than homodimers and furthermore that heterodimer formation has a growth inhibitory effect in breast and prostate epithelial cells [124]. Previous studies by Powell et al. [46] showed that the ERβ selective agonist, liquiritigenin, which can bind to both ER subtypes, induces an ERα conformation that prefers heterodimerization with ERβ, as opposed to forming ERα homodimers. Therefore, we cannot exclude heterodimer formation as an explanation for the strong agonist effect of the *Cyclopia* extracts in the transactivation model in MCF-7BUS cells.

Having evaluated the agonist and antagonist activity of *Cyclopia* extracts in a system where the ER subtypes were expressed separately and together, in a transactivation and a transrepression model, we increased the level of complexity by evaluating the effect of the extracts on MCF-7BUS cell proliferation, a system where the final cell phenotype is a product of not only the two ER subtypes but also of an integrated transactivation and transrepression system [39,98-100]. Although the *Cyclopia* extracts, like E_2, induced cell proliferation it was with either a significantly lower potency (P104 and SM6Met) or lower efficacy (P104 and cup-of-tea)

than E_2. Furthermore, in the presence of E_2, all of the *Cyclopia* extracts displayed antagonistic properties. Similarly, the polyphenols also induced cell proliferation with either lower efficacies or potencies than E_2 and some (genistein and enterodiol) also displayed antagonism. Previously, the agonist activity seen in the transactivation model in MCF-7BUS cells was ascribed to ERβ activation and this is probably translating into weak induction of MCF-7BUS cell proliferation. Furthermore, liquiritigenin, an ERβ selective agonist, although not able to induce significant MCF-7 cell growth in a mouse xenograft model [19,24], was able to induce proliferation of the ERα and ERβ positive [125] osteoblast-like murine MC3T3-E1 cells [126]. The antagonism of E_2-induced cell proliferation by extracts of *Cyclopia* could be attributed to ERα antagonism (observed in the transactivation model in COS-1 cells), ER mediated repression of proliferation inducing genes (ER transrepression observed in MCF-7BUS transrepression model), ERβ-mediated transcription (observed in the transactivation model in COS-1 cells) of anti-proliferative and anti-apoptotic genes [39,127], or they might favor the formation of ERα/β heterodimers, which has been suggested to have growth inhibitory effects in breast epithelial cells [124].

Furthermore, we also evaluated the estrogenic and antiestrogenic properties of the *Cyclopia* extracts in an *in vivo* model, an immature rat uterotrophic assay. For the first time we show *in vivo* biological activity of the phytoestrogenic extracts of *Cyclopia*. SM6Met and cup-of-tea, unlike E_2 and genistein, did not increase uterine weight and SM6Met, like the ER antagonist ICI 182,780, antagonized E_2-induced uterine growth. The ERα subtype is the major subtype expressed in the uterus with very low levels of ERβ expressed [56,101]. Powell et al. [46] show that although ERβ homodimers and ERα/ERβ heterodimers are favored, genistein is capable of inducing ERα homodimers and activating ERα-induced transcription. Therefore, we can assume that the increase in uterine growth induced by genistein in the uterotrophic assay is a product of increased ERα homodimerization and hence, increased ERα mediated transcription. The ERβ selective agonists, liquiritigenin and ERB-041, in contrast, do not induce uterine growth [19,128]. Thus, the findings regarding ERβ selective agonists combined with the low levels of ERβ in the uterus excludes ERβ as the subtype eliciting the effect of *Cyclopia* extracts in the uterus. It is thus likely that the effect of *Cyclopia* extracts is due to ERα antagonism, as seen in the transactivation model in COS-1 cells, or that upon binding to the ER, the *Cyclopia* extracts induce a change in conformation that inhibits co-activator recruitment or activates co-repressor recruitment. The inability of the *Cyclopia* extracts to induce uterine growth, in contrast to MCF-7BUS cell proliferation, might also be attributed to either the differences in the concentration of co-regulators or the differences in co-regulator recruitment in the breast and uterus [129,130].

Having established ER agonist and/or antagonist activity of *Cyclopia* extracts, we look towards HPLC data, from the current and previous studies, to identify the polyphenol(s) responsible for the observed effects. The xanthones, mangiferin and isomangiferin, were identified in all *Cyclopia* extracts, but as mangiferin has no estrogenic potential, while isomangiferin has not previously been tested for estrogenicity [71], it is unlikely that the observed ER agonist/antagonist effects of *Cyclopia* can be ascribed to these polyphenols. However, mangiferin has been shown to inhibit the proliferation of breast cancer cells via ER independent mechanisms [131] and therefore, as mangiferin is present in all extracts at relatively high amounts it cannot be excluded as the polyphenol antagonizing E_2-induced MCF-7BUS cell proliferation. Of the remaining polyphenols identified in the extracts the only aglycone present is the flavone, luteolin. *In vitro*, luteolin binds to both of the ER subtypes, is an ERα and ERβ agonist, induces MCF-7BUS cell proliferation, and antagonizes E_2-induced MCF-7BUS cell proliferation [62,63,71,132-134]. Therefore, with regards to the *Cyclopia* extracts, the ERβ agonism observed in the transactivation model, the induction of MCF-7BUS cell proliferation, and the antagonism of E_2-induced cell proliferation may be ascribed to the presence luteolin in the extracts, however, the observed ERα antagonism in the transactivation model cannot. Although luteolin is present in all extracts, the concentration is low. However, the 7-*O*-rutinoside of luteolin, scolymoside, is present in substantial amounts in all of the *C. subternata* extracts (presence was not evaluated in P104). This rutinoside of luteolin has not previously been tested for estrogenicity [71], however, as glycosides may be hydrolyzed by intestinal β-glucosidases [135,136], the bioavailability of the aglycone, luteolin, and hence phytoestogenicity of the extracts may increase upon hydrolysis of scolymoside. Furthermore, luteolin has been shown to have anti-tumor characteristics and can sensitize breast cancer cells to anti-tumor drugs such as tamoxifen [137] and therefore, the presence of luteolin, as well as scolymoside, in *Cylopia* extracts can be seen as positive regarding chemoprevention as well as breast cancer treatment. Generally, the glycosides of polyphenols either display reduced estrogenic activity compared to the aglycones or have not been evaluated for estrogenicity [71]. Thus, if the hydrolysis of glycosides present in the *Cyclopia* extracts is considered, it allows us to evaluate the phytoestrogenicity of the aglycones alongside their glycosides: apigenin (aglycone of vicenin-2), eriodictyol (eriocitrin), hesperitin (hesperidin), phloretin (phloretin-3,5-di-C-glucoside), hydroxyphloretin (3-hydroxyphloretin-3',5'-di-*C*-hexoside), and iriflophenone (iriflophenone-2-*C*-β-glucoside and iriflophenone-di-*O,C*-hexoside). However, as β-glucosidases are produced by intestinal flora [138,139], consideration of glycoside metabolism will not help to identify the polyphenols responsible for *in vitro* results but may only be relevant for interpretation of *in vivo* results. For example, as luteolin and apigenin have been shown to significantly increase uterine weight, either in the presence or absence of estrogens [140,141], the effect elicited by *Cyclopia* extracts *in vivo* cannot be ascribed to luteolin, scolymoside, or vicenin-2. The effect of the other identified polyphenols has not been evaluated *in vivo* and therefore we cannot definitively attribute the *in vivo* effect of the *Cyclopia* extracts to any of these polyphenols. Of the glycosides, ericotrin and hesperidin have been tested for phytoestrogenicity *in vitro* [71]. However, hesperidin does not bind to the ER [62] or activate an ERE-containing promoter reporter construct [133]. Eriocitrin, however, has been shown to

bind to only ERβ [62], but no work has been done to elucidate the estrogenic effect elicited by this polyphenol. For the first time we identified the dihydrochalcone, aspalathin, in *Cyclopia*. Aspalathin has not been tested for estrogenicity but has been shown to inhibit the proliferation of liver cells [142], however, due to the presence of unique drug metabolizing enzymes in the liver, the possibility of aspalathin metabolites eliciting this effect cannot be excluded nor can the results be extrapolated to breast cancer cells. The phytoestrogenicity of the remaining glycosides and aglycones, as well as protocatechuic acid, has not been tested [71]. In summary, none of the compounds identified in the *Cyclopia* extracts can account for the observed ERα antagonism, some (luteolin and eriocitrin) may explain the observed ERβ agonism and others (mangiferin and aspalathin) should not be excluded as possible effectors of ER-independent effects on proliferation. Therefore, thus far, we cannot with certainty ascribe the effects observed with *Cyclopia* extracts in this study to any of the individual constituents of our extracts. Although, further research regarding the polyphenol content, bioavailability, and estrogenic activity of our extracts is required to identify the compound causing the observed effects, we cannot exclude the possibility that a mixture of polyphenols is required to elicit the effects observed with *Cyclopia* extracts.

Physiologically, our results may be assessed both in terms of treatment of menopausal symptoms (hot flashes, osteoporosis, and increased inflammation [2-4,52,90-92]) and prevention of estrogen replacement associated side effects (breast cancer and uterine proliferation [5,6,52]). With regards to menopausal symptoms, the ERβ agonist MF101 [24], has been shown in clinical trials to reduce hot flashes and thus, the ERβ agonism of the *Cyclopia* extracts may be considered as a positive attribute. Furthermore, with regards to the postmenopausal surge in inflammatory disorders the fact that the *Cyclopia* extracts displayed agonism in the transrepression model in MCF-7BUS cells may also be considered as a positive attribute for the treatment of postmenopausal inflammatory disorders. With respect to the known roles of ER subtypes in breast cancer [15,22,37-43], the fact that extracts of *Cyclopia* antagonize ERα, while being ERβ agonists, may be beneficial. In addition, the extracts were able to antagonize the proliferation of breast cancer cells in the presence of E_2 at lower concentrations than that required for breast cancer cell proliferation. Furthermore, not only do the *Cyclopia* extracts show potential as protectors against breast cancer development and inflammatory disorders, they also do this without promoting uterine growth, a negative SERM associated side effect [35,143].

Although *Cyclopia* extracts show potential to be developed as SERSMs, further work, which is ongoing, is needed to clarify their mechanism of action. This includes, but is not limited to, directly comparing the *Cyclopia* extracts with the known SERMs tamoxifen and raloxifene, investigating the effect of *Cyclopia* extracts on ER subtype levels, ER homo- or heterodimerization, induction or inhibition of co-regulator recruitment, and the modulation of cancer development and progression in a rat breast cancer model. In addition, further work is needed to identify the polyphenol(s) responsible for eliciting the observed effects and the possibility that distinct polyphenols present in *Cyclopia*, rather than an individual polyphenol, may be causing the observed ERα agonism and ERβ antagonism cannot be excluded.

Supporting Information

Figure S1. Determination of ERE-containing promoter reporter construct concentration. (A & B) COS-1 cells, transfected with equal amounts of (A) ERα and (B) ERβ, and (C) MCF-7BUS cells were transfected with increasing amounts of the ERE-containing promoter reporter construct (ERE.vit2.luc) and treated with either solvent or E_2 to determine at which concentration of the ERE-containing promoter reporter construct the highest induction of E_2 is observed. The dotted line through the bars represents the values for solvent control. Fold induction is indicated in boxes above the E_2 columns. Average ± SEM is of one experiment done with three to four repeats.
(TIF)

Figure S2. Determination of NFκB-containing promoter reporter construct concentration. (A & B) COS-1 cells, transfected with equal amounts of (A) ERα and (B) ERβ, and (C) MCF-7BUS cells were transfected with increasing amounts of the NFκB-containing promoter reporter construct (p(IL6kB)350hu.IL6Pluc+) and treated with either solvent, PMA or PMA + E_2 to determine at which concentration of the NFκB-containing promoter reporter construct the highest repression by E_2 of PMA induction is observed. The dotted lines through the bars represent the values for either solvent control or 10ng/ml PMA. Percentage repression, where applicable, is indicated in boxes above the PMA + E_2 columns. Average ± SEM is of one experiment done with three repeats.
(TIF)

Figure S3. The effect of the SM6Met and cup-of-tea extracts on immature rat uterine growth. Immature female wistar rats were treated with 2000, 200, and 20mg/kg body weight SM6Met and cup-of-tea for three consecutive days. Animals were sacrificed on day four, (A) uteri were photographed and (B) wet and (C) blotted uterine/final body weight was determined. One-way ANOVA with Dunnett's post-test comparing all columns to solvent control (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). The dotted line through the bars represents the values for solvent control. Average ± SEM is of at least eight animals/group.
(TIF)

Figure S4. The effect of E_2, genistein, extracts of *Cyclopia*, and ICI on body and liver weight. Immature female wistar rats were treated for three consecutive days with 100µg/kg body weight (BW) E_2, in the presence and absence of 2000mg/kg BW SM6Met or 10mg/kg BW ICI 182,780, 100mg/kg BW genistein, 2000, 200, or 20mg/kg BW SM6Met, 2000, 200, or 20mg/kg BW cup-of-tea, and 10mg/kg BW ICI 182,780 for three consecutive days. Animal were sacrificed on day four and changes in (A) body and (B) liver weights were

determined. One-way ANOVA with Dunnett's post-test comparing all columns to solvent control (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control (A and B) and 100µg/kg BW E_2 (A). Average ± SEM is of at least five animals/group.
(TIF)

Figure S5. The effect of different concentration of the SM6Met and cup-of-tea extracts on the onset of vaginal opening. Immature female wistar rats were treated for 30 consecutive days with the SM6Met and cup-of-tea extracts and the day of vaginal opening was determined. One-way ANOVA with Dunnett's post-test comparing all columns to solvent control (*, P<0.05; **, P<0.01; ***, P<0.001). The dotted line through the bars represents the values for solvent control. Average ± SEM is of at least eight animals/group.
(TIF)

Table S1. Summary of ER agonism and antagonism of *Cyclopia* extracts.

(DOCX)

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Author Contributions

Conceived and designed the experiments: AL KV. Performed the experiments: KV MM. Analyzed the data: AL KV MM. Contributed reagents/materials/analysis tools: AL. Wrote the manuscript: AL KV.

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