THE AGE-OLD PROBLEM OF POLLUTION, ITS ROLE IN ENDOCRINE DISRUPTION AND THE CURRENT ANALYTICAL TECHNOLOGIES THAT CAN BE EMPLOYED TO MONITOR AND ASSESS WASTE WATER TREATMENT PLANTS

by
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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Daniel Wilhelm Olivier

March 2017

There is no bigger honor than to dedicate my thesis to my grandparents, oupa Boggom and ouma Kaap, who I lost while in the process of writing this thesis. You will forever be in my memories.

“Ek kan, ek wil, ek sal!”

Summary

Water scarcity is a global problem and pollution of this valuable resource is a growing concern. South Africa is no exception. As part of an on-going study aimed at developing decentralized water treatment systems based on biomimicry design, this project aimed to evaluate and optimize analytical methods that can be used to evaluate the efficiency of these systems in removing compounds with endocrine disrupting properties. In addition, this project also aimed to show the possible consequences if pollutants are not removed by investigating the effects of a number of endocrine disrupting chemicals in combination with one another and in combination with natural human hormones. This should provide a more realistic view of how a combination of pollutants that typically end up in the environment due to pollution can adversely affect organisms, and by extension, the ecosystem. It should also contribute to our understanding of how common pollutants that gets applied on the skin as personal care products (PCPs) can possibly influence human health and be linked to diseases such as breast cancer.

The first aim resulted in a method that can be used to isolate, identify and quantify 11 endocrine disrupting chemicals (3 hormones, 1 synthetic hormone analog, 4 PCPs, 2 plasticizers and 1 anticonvulsant) and one human indicator. The method uses solid phase extraction to isolate compounds, dansyl chloride derivatization of compounds to enhance mass spectrometry detection and a novel super-critical fluid chromatography system, called an ultra-performance convergence chromatography (UPC^2) system, coupled to tandem mass spectrometry for detection and quantification of each compound. This method can be used to evaluate the removal efficiency of waste water treatment systems. However, method validation revealed additional optimization and simplification should be considered. The second aim yielded data that showed the combined effect four common PCP pollutants as either being additive, antagonistic or synergistic. The data highlights how these PCPs can possibly interfere with the endocrine system of humans and animals if used as PCPs or are found in environment as pollutants. Finally, the data also suggest how common PCPs can influence diseases such as breast cancer.

Opsomming

Water skaarste is ‗n wereld-wye probleem en die besoedeling van hierdie belangrike hulpbron is ‗n toenemeende bekommernis. Suid-Afrika is geen uitsondering. As deel van ‗n voortgaande studie met die doel om gedesentraliseerde water sisteme wat gebaseer is op biomimieke beginsels te ontwikkel, het hierdie projek beoog om die analitiese metodes wat gebruik kan word om hierdie sisteme se bekwaamheid in die verwydering van endokrien ontwrigtende produkte te ondersoek te evalueer en te optimiseer. Daarmee saam het hierdie projek ook beoog om die moontlike nadelige effekte van besoedeling te ondersoek deur ondersoek in te stel op ‗n kombinasie van endokriene ontwrigters met mekaar, asook in kombinasie met natuurlike menslike hormone. Hierdie behoort ‗n realistiese oorsig te gee oor hoe algemene produkte wat water bronne besoedel in kombinasie organismes ongunstig kan affekteer en so ook die ekosisteem. Dit sal ook bydra tot huidige kennis oor hoe algemeen besoedelende produkte wat op die vel aangesmeer word as persoonlike hieniese produkte (PHPs) die mens se gesondheid kan affekteer en verbind kan word tot siektes soos bors kanker.

Die eerste doelwit het ‗n metode opgelewer wat 11 endokriene ontwrigters (3 hormone, 1 sintetiese hormoon anoloog, 4 PHPs, 2 plastiseerders en 1 antistuipgif) en een menslike indikator kan isoleer, identifiseer en kwantifiseer. Die method maak gebruik van soliede fase ekstraksie vir die isolering van produkte, dansiel chloried derivatisering van produkte vir verbeterde massa spektrometrie deteksie en ‗n nuwe super-kritiese vloeistof kromatografie, bekend as ‗ultra-performance convergence chromatrogarphy (UPC^2)‘, gekoppel aan aaneengekoppelde massa spektrometrie vir die deteksie en kwantifisering van elke produk. Hierdie metode kan gebruik work om die verwyderings bekwaamheid van afval-water behandeling sisteme te evalueer. Metode bevestiging wys wel dat aandag gegee sal moet word aan verdere optimisering en vereenvoudiging van die metode. Die tweede doelwit het bewys dat die effek van kombinasies van PHP besoedelende produkte sommerend, antagonisties of sinergisties kan optree. Die data wys verder die moontlike effekte wat PHPs kan hê op die endokriene sisteem van mense en diere as hierdie produkte gebruik word as PHPs of as besoedelende produkte in die natuur voorkom. Laastens, wys die data ook hoe algemeen gebruikte PHPs siektes soos bors kanker kan beïnvloed.

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UPC^2-MS/MS method validator. Your effort and time cannot be quantified! Finally, thank you for just being you…I knew I made the right choice when I asked you out many, many moons ago. I love you double-infinity.

List of abbreviations

4NP	4-Nonylphenol
AhR	Aryl hydrocarbon receptor
AKR	Aldo-keto reductase
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BC	Breast cancer
BOD	Biological oxygen demand
BPA	Bisphenol A
BSTFA	*N,O*-Bis(trimethylsilyl)trifluoroacetamide
CAR	Constitutive androstane receptor
CBZ	Carbamazepine
CCD	Catalytic combustion detector
CE	Collision energy
CI	Chemical ionization
COD	Chemical oxygen demand
CPRG	Chlorophenol red-β-D-galactopyranoside
CV	Collision voltage
CYP	Cytochrome P450s
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DID	Discharge ionization detector
DNCl	Dansyl chloride
E_1	Estrone
E_2	17β-Estradiol
E_3	Estriol
EC_{25}	Potency of a compound at 25% of the Max E_2 response

EC_{50}	Potency of a compound at 50% of the Max E_2 response
EC_{75}	Potency of a compound at 75% of the Max E_2 response
ECD	Electron capture detector
ED	Endocrine disruption
EDC	Endocrine disrupting chemical
EE_2	17α-Ethinyl estradiol
EI	Electron ionization
EM	Electron multiplier detector
EM	Experimental medium
ER	Estrogen receptor
ESI	Electron-spray ionization
FID	Flame ionization detector
FTICR	Fourier transform ion cyclotron resonance detector
GC	Gas chromatography
GC	Gas chromatography
GLPC	Gas-liquid partition chromatography
GM	Growth medium
GR	Glucocorticoid receptor
HID	Helium ionization
HPA	Hypothalmic-Pituitary-Adrenal
HPG	Hypothalmic-Pituitary-Gonadal
HPLC	High performance liquid chromatography
HPT	Hypothalmic-Pituitary-Thyroid
HRT	Hormone replacement therapy
HSD	Hydroxysteroid dehydrogenase
IBCERCC	Interagency breast cancer & environmental research coordinating commission

IRD	Infrared detector
iXRE	Inhibitory xenobiotic response element
JTED	John Todd Ecological Design
LC	Liquid chromatography
LC	Liquid chromatography
MAA	Methoxy acetic acid
MALDI	Matrix-assisted laser desorption ionization
MAPK	Mitogen-activated protein kinase
MCF-7 (BUS)	Mitchigan Cancer Foundation 7 (BUS)
MeP	Methyl parabens
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometer detector
MS/MS	Tandem mass spectrometry
NIH	National Institute of Health
NOEL	No observable effect level
NPD	Nitrogen-phosphorus detector
NR	Nuclear receptor
OH-TAM	Hydroxy-tamoxifen
PAH	Poly aromatic hydrocarbons
PCB	Polychlorinated bephenyl
PCM	Paracetamol
PCPs	Personal care products
PDA	Photo diode array
PDD	Pulsed discharged ionization detector
PID	Photo-ionization detector
PPAR	Peroxisome proliferator-activated receptor

PPCPs	Pharmaceuticals and personal care products
PR	Progesterone receptor
PrP	Propyl parabens
PXR	Rodent prenane X receptor
RXR	Retinoid X receptor
SFC	Super critical fluid chromatography
SRD5A	5α-Reductase
SRM	Single reaction monitoring
SXR	Human steroid and xenobiotic receptor
T	Testosterone
T_2	Iodothyronine
T_3	Tri-iodothyronine
T_4	Thyroxine
TAM	Tamoxifen
TBT	Tributyltin
TCC	Triclocarban
TCD	Thermal conductivity detector
TCS	Triclosan
TF	Transcription factor
TOF	Time of flight
TQ-S	Tandem quadrupole S-wave
TR	Thyroid receptor
UPC^2	Ultra-performance convergence chromatography
UPLC	Ultra performance liquid chromatography
USEPA	United States Environmental Protection Agency
UV	Ultra violet
VUV	Vacuum ultraviolet detector

WC	Western Cape
WWTP	Waste water treatment plant
YAS	Yeast androgen screen
YES	Yeast estrogen screen

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CHAPTER 1

Thesis overview

1.1 General background

1.1.1 This project forms part of a bigger study that aims to solve a current water pollution problem

In South Africa, most of the freshwater is supplied by the Orange, Mzimvubu, Mvoti, Thukela, Usutu, Inkomati and Vaal Rivers, while the Olifants, Luvuvhu/Letaba, Fish, Gouritz, Breede and Berg rivers contribute to a lesser extent (1). Of these thirteen rivers the Gouritz, Olifants, Breede and Berg rivers supply the Western Cape (WC) with freshwater. In early 2011, it came under the WC's department of environmental affairs and development plannings (DEA & DP) attention that untreated effluent water (greywater) from two informal settlements (Langrug outside Franschoek, and MBekweni near Paarl) were running directly into the Berg River. A study by Royden-Turner (2) found that the effluent greywater from these settlements had high microbial counts and were highly concentrated in pharmaceutical and personal care products (PPCPs).

Agriculturally important for irrigation, contamination of the Berg River could lead to a decrease in exported crops due to presence of microbes and pollutants such as PPCPs in irrigation supplies. This in retrospect will then negatively influence the economy. Residents of both Langrug and Mbekweni heavily depend on the economy of the Cape Winelands district, of which Franschoek and Paarl form part of, as most of these residents are seasonal farmworkers. Therefore, the health status of the Berg River is of the utmost importance to residents depending on its water supply. In addition, the river is also important for wildlife living in its vicinity.

In 2013 John Todd Ecological Design (JTED) partnered with Biomimicry SA, Informal South^{TM}, Greenhouse^{TM} and Maluti Waters^{TM} to develop an onsite, eco-friendly, natural treatment system that will firstly, treat the effluent greywater from Langrug informal settlement, and secondly, create jobs to uplift the community. A pilot scale model of the developed biomimicry based treatment system, called the JTED Eco-machine, suggested effective treatment of greywater. However, the treatment of PPCPs was not investigated and the efficiency of such a system will only be known through testing of the actual system (3, 4).

Evaluating/Monitoring the efficiency of a system such as the JTED Eco-machine requires a trans-disciplinary approach involving amongst others microbiologists, biochemists, ecologists and engineers, considering the complexity of such a system, as the hydraulics, the effect of microbial communities (e.g. biofilms) and the health of the fauna and flora are equally important. Hence, the bigger picture of a study such as the one this project forms part of provides unique challenges.

1.1.2 Evaluating the JTED Eco-machine: from a biochemistry perspective

From the study by Royden-Turner followed an inquiry into the ability of the JTED Eco-machine to remove PPCPs. Considered as emerging contaminants (5, 6), PPCPs can elicit certain biological responses in humans and animals coming in contact with water polluted with these compounds and therefore they pose a health risk. Some PPCPs have been linked to causing and contributing to endocrine disruption (ED) – a phenomenon of great importance to biochemists and physiologists that will be discussed in more detail in chapter 2 section 2.5.5. Together with personal and family history of cancer, the use of alcohol, smoking and a number of other consequences, ED is thought to be the root of diseases such as obesity, diabetes and cancer. Therefore, the JTED Eco-machine needs to be evaluated for its ability to remove PPCPs. To achieve this, methods would need to be in place that will enable the pre-concentration, detection and quantification of PPCPs.

1.1.3 Methods that can be employed to measure the presence of PPCPs

Over the past century numerous methods have been developed to detect compounds such as PPCPs in different matrices such as the environment, blood, urine and breast milk. Gas chromatography (GC), liquid chromatography (LC) and supercritical fluid chromatography (SFC) all coupled to mass spectrometry are the most noteworthy and will be discussed in chapter 2 section 2.4. Alternatively, methods have also been developed to show the presence of a group of compounds, e.g. endocrine disrupting chemicals (EDCs), in a sample. However, here a biological response is tested indicative of the members in that specific group rather than the individual compounds. Noteworthy of these methods are the yeast estrogen screen (YES) and E-SCREEN, which will be discussed in chapter 2 section 2.3. In addition to their ability to show the presence of a group of compounds in a sample, these biological assays can also be used to investigate the modes of action (MOA) of EDCs. This added benefit will be highlighted in chapter 2 section 2.3 and their use become apparent in chapters 4 and 5 when the aforementioned assays are used investigate the risks associated with the presence of certain antimicrobials present in personal care products (PCPs) that find their way into the environment.

1.2 Objectives of this study

The overall goal of this study was to find the most suitable chromatography method that could be used to evaluate the JTED Eco-machine‘s ability to remove pollutants such as PPCPs. In addition, the study aims to substantiate the possible consequences to humans and animals if exposed to pollutants with ED abilities from contaminated water resources.

The specific objectives were to:

a) Develop analytical techniques for the pre-concentration, identification and quantification of 11 commonly occurring pollutants with ED capabilities from environmental matrices (CHAPTER 3).
b) Elucidate the pharmacological property – drug potency, of four antimicrobials and three human estrogens on the YES and E-SCREEN (CHAPTER 4).
c) Investigate the combinatory effects of PCPs and their possible contribution to breast cancer (CHAPTER 5).

Chapters 4 and 5 use breast cancer (BC) as an example of the possible consequence of EDC exposure to humans.

CHAPTER 2

Literature review

2.1 Introduction

Chapter 1 provided a short history on how this project came into play, touched on some of the most important topics that will be discussed and dealt with throughout the rest of this thesis and supplied the overall goal and specific objectives set out for this study. This chapter aims to provide additional or a deeper knowledge into some of the subjects that were touched on. Chapter 2 is divided into three additional sections. The second section will deal with the global problem of water pollution and the issue at hand in the Berg River, as well as the methods that can be used for monitoring pollution. Together, these topics aim to provide information relevant to why the current study is needed and a background into the methods used in chapter 3 that can be used to evaluate the JTED Eco-machine. The third section will focus on two screens, which like the chromatographic techniques discussed in topic 2c, can be used to monitor pollution. However, here the two screens will be discussed as bioassays that can be used to investigate endocrine modulation. The aim of this section is to provide background information into the bioassays as they are used in chapter 4 and 5 to emphasize the affect that certain pollutants can have on the endocrine system of humans and animals. However, special regard will be given to humans as the pollutants in question are thought to also contribute to ED mediated breast cancer. From this, parallel conclusions can be drawn on their possible effects on wildlife in the vicinity of the Berg River, their possible effect on crop export and evidently the residents of the two informal settlements if present in the Berg River. Finally, section four will provide background information into the human endocrine system and its role in the regulation of homeostasis, how pollutants such as PPCPs with ED capabilities find their way into the human body, their modes of ED and current data on the most prominent EDCs of concern. The aim of this final section is to highlight how important the clean-up of a river such as the Berg River is and the diverse consequences that can follow if not. In addition, the section will summarise the current state of EDC research and serves as supporting information that the compounds investigated in chapter 4 and 5 are reason for concern.

2.2 Water pollution

In 2000 the United Nations set out eight Millennium developmental goals (MDG‗s) – one of which was to ensure environmental sustainability – with water availability being a primary concern. According to the 2015 MDG report, ‗water scarcity affects more than 40% of the global population and is projected to rise', with Northern Africa and parts of Western Asia already exceeding sustainable limits. Nonetheless, places such as Eastern Asia, South-Eastern Asia, Latin America and the Caribbean, Oceania and Sub-Saharan Africa have adequate water resources, but in some of these regions, such as Africa face a different problem: inadequate sanitation services – a problem that may threaten water resources. With up to 62% of the urban population living in slums, having insufficient waste removal system, waste water is discarded outside homes creating unsanitary living conditions. In most cases this waste water runs off into smaller streams that eventually end up in rivers, lakes, ponds or dams, contaminating the remaining freshwater supply (7).

South Africa‗s water vulnerability is no exception and the pollution of the Berg River is a typical example. Alongside the Berg River two informal settlements are threatening the health of the river as household waste water from these settlements is draining into the river. As the Berg River is one of the primary rivers used for irrigation in the Cape Winelands, pollution of the river could have dire consequences. These include possible reduced crop export due to high microbial and PPCP loads from crops irrigated with water from the Berg River and the health of the ecosystems surrounding the river. As the agricultural industry in the areas surrounding and supplied by the Berg River heavily depend on exports and tourism, reduced export of crops or tourism could in retrospect affect the livelihood of those making a living from these sectors. As a large portion of the population is seasonal farmworkers, living in these informal settlements, these residents stand to lose the most.

In an effort to save the river, the agricultural and tourism industry and the livelihood of those that could be affected from a declining economy, a number of organisations worked together with the WC‗s DEA & DP to solve this growing concern. The proposed solution, called the JTED Eco-machine (Figure 2.1), operates on biomimetic principles. In short, these biomimetic principles are in fact the genius of nature itself, where humans learned how nature solves problems and then imitated them. These principles are encapsulated in the field of biomimicry. Good examples where biomimicry has been used are bioSTREAM, bioWAVE and Sharklet antimicrobial surfaces. The first two technologies are reverse-engineered technologies imitating the efficiency of the tail of a tuna fish to create energy. Sharklet technologies in contrast imitated the antimicrobial surface of sharkskin to create antimicrobial surface coverings that can be used in hospitals.

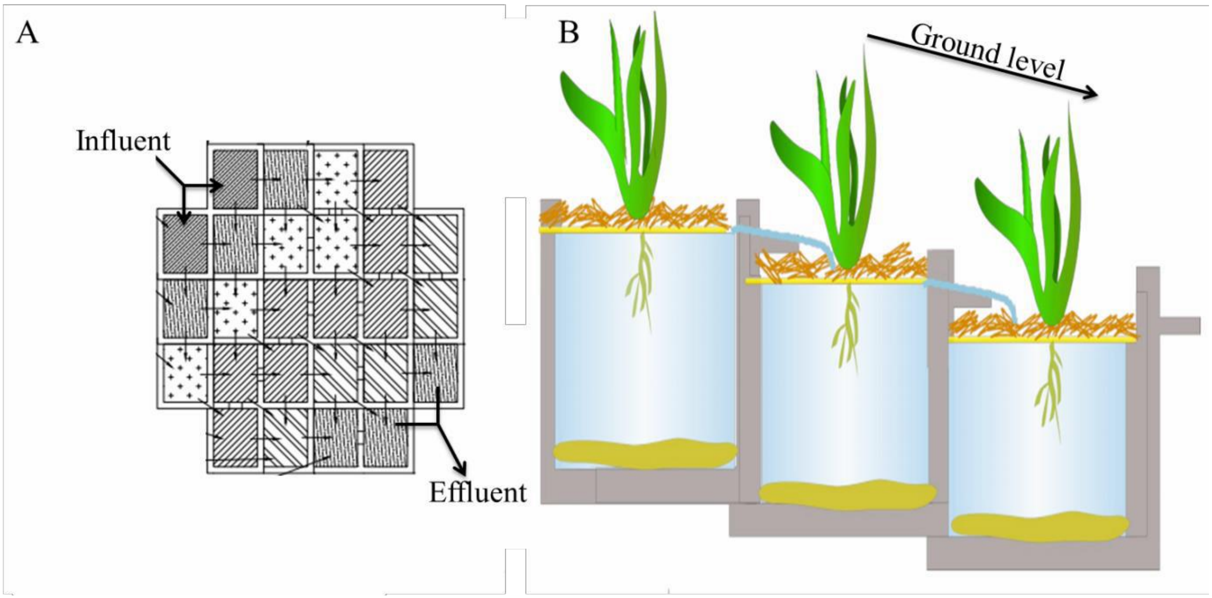

Figure 2.1 The JTED Eco-machine. A) Top-view of one of three 26-cell cascades showing the influent and effluent points. After the third cascade water flows onto an algal turf scrubber (not shown). B) Side view of three cells of a 26-cell cascade, showing how water will be treated and the natural flow of the water downwards.

The JTED Eco-machine aims to treat greywater from the informal settlements using the biomimetic principles in two stages. First, recipient greywater is cascaded through multiple aquatic cells, wherein the water is slowed down and sped up sequentially, allowing sufficient retention time for biological degradation of solid waste and the uptake and metabolism of dissolved or suspended substances by a diverse set of microbial, plant and animal species. The cascading aquatic cells work on the dendritic principle, whereby each cell feeds two or more cells downstream, distributing load and effectively filtering the water from one cell to the next, before converging to one filtered mass of water. During the final stage of treatment the filtered water is scrubbed from remaining nitrates, nitrites, ammonia, ammonium and phosphates by an algae turf scrubber before being released into the environment (2).

As mentioned in section 1.1.1, a pilot scale study found that greywater was treated efficiently but the study did not include ability of the Eco-machine to remove PPCPs and that its robustness (section 1.1.2) will only be evident from the actual system under real conditions. Henceforth, the appropriate PPCP monitoring methods would need to be in place if the PPCP removal efficiency is to be determined.

2.3 Biological methods employed to monitor pollution

2.3.1 Biological methods overview

To date several biological methods have been validated to investigate pollution (Table 2.1). Most of these methods relate to water pollution.

Table 2.1 Validated methods used to investigate pollution. Adapted from P Kumar (8).

General bio-assays	Cell bioassays	Molecular bioassays	Biosensors
Plant	Membrane damage	DNA probes	Biological/chemical oxygen deman. (BOD/COD)
Algal	Cytogenicity	Immuno assays	Microbial gas
Vascular macrophytes	Sister chromatid exchange	Bioluminescence Lux-reporter genes	Immuno assays
Mosses	Micronucleus test	Yeast estrogen screen (YES)	
Lichens	Ames test	Yeast androgen screen (YAS)	
Pollution-induced peptides	Chromosomal damage		
Fish	E-SCREEN (MCF7 proliferation)		
Protozoa			
Helminthic			

In the past 2 decades the focus of pollution has shifted from general pollution to specific pollution, with the use of assays such as the YES/YAS and E-SCREEN describing rather the type of pollution than just pollution in general. Specifically, the focus has shifted to pollution that can disrupt the homeostasis of organisms such as the type of pollution that can disrupt the endocrine system of mammals, termed endocrine disruption, leading to adverse side effects (e.g. feminization of male fish). For the purpose of this thesis the YES and E-SCREEN assays will be used as examples of how pollution can be monitored with biological methods. Both methods are used to test for pollution that can cause ED in mammals and are used in chapters 4 and 5.

2.3.2 The yeast estrogen screen (YES)

The YES was originally developed by Routledge and Sumpter (9) to detect compounds in water with estrogen-like properties. Positive results from a sample subjected to the assay indicate that under cellular conditions the sample has the ability to stimulate the estrogen receptor (ER). As these receptors play an intricate role in regulating cellular processes (discussed in section 2.5.4), unwanted stimulation of these receptors could disrupt homeostasis. This is then indicative of a sample that contains pollutants able to cause ED.

In the assay (Figure 2.2), yeast stably expresses the human ERα. In the presence of an ERα agonist, the ERα-ligand complex binds to the ERE of an ERE-containing plasmid where-after TF's and other transcriptional components are recruited for transcription. Following this, the transcriptional complex transcribes the gene *Lac-Z*, coding for β-galactosidase. The latter is secreted into the medium where it metabolizes chlorophenol red-β-D-galactopyranoside (CPRG) – a chromogenetic substrate, causing a colour change from yellow to red. The change in colour is then measured spectrophotometrically.

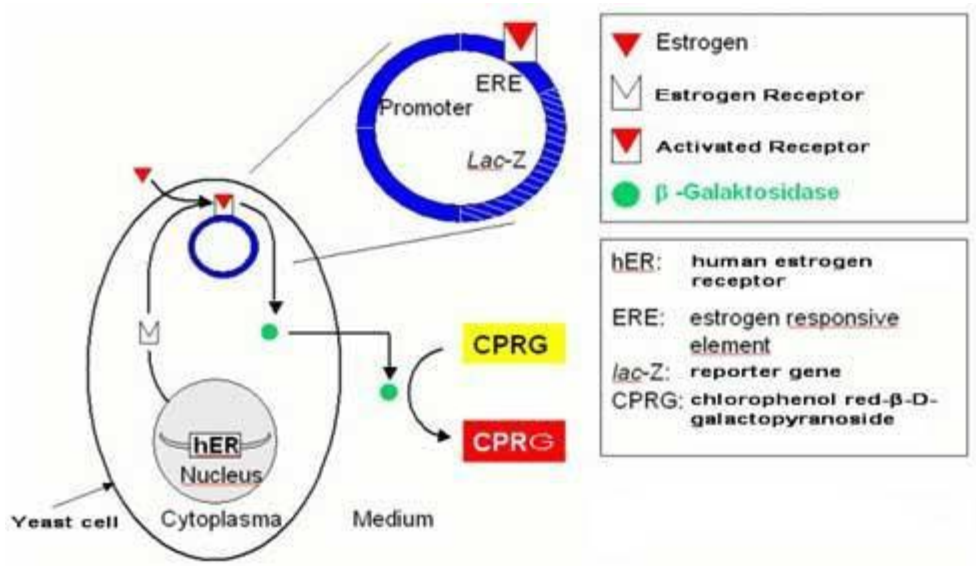

Figure 2.2 Yeast estrogen screen (YES) assay used for observing modulatory effects mediated through ERα.

In addition to its ability to serve as an ED pollution indicator, this screen has also proven useful in determining dose-response parameters (e.g. potency and efficacy) of certain estrogen-like compounds. These include environmental estrogens (10), surfactants (9), natural and synthetic estrogens, pharmaceuticals, antimicrobials and alkylphenols (11), flavonoids (12), food contact materials and dichlorobenzenes (13), among other chemicals. The benefit of using this assay is that modulation of ERs can be viewed in isolation, without the interference of other cellular signal transduction pathways (see section 2.3.2), such as the case with cellular assays (e.g. the E-SCREEN).

2.3.3 The E-SCREEN

Round about the same time as Routledge and Sumpter developed the YES, Soto *et al.* (14) developed the E-SCREEN to evaluate the estrogenicity of environmental compounds as an alternative method to the then used rodent assay. The screen makes use of Michigan cancer foundation 7 (MCF-7) breast cancer cells [ERα positive cells, (15)] and tests the ability of a compound to cause the cellular proliferation of these cells. However, in recent years a modified form of the MCF-7 cells, MCF-7 BUS cells, has been used in the E-SCREEN. These cells provide a higher proliferative response than normal MCF-7 BC cells (16) and therefore increases the sensitivity of the assay.

In the original assay MCF-7 cells are cultured before being plated in wells. Plated cells are allowed to attach overnight before the seeding medium is removed and replaced with experimental medium. Test compounds are then added to the experimental medium at a range of different concentrations and incubated for a number of days. After terminating the assay by removing the medium, cells are lysed and the nuclei counted. The range of concentrations and their nuclei counts then provide information on the proliferative effects of the compound. However, modifications to the screen such as the method used to quantify proliferation (12, 17), seeding density and preconditioning of cells with experimental medium (18) have previously been done. To date the E-SCREEN has been used among other things to detect estrogenic pollution in water resources (19), bottled water (17), characterizing estrogenic potential of flavonoids (12) and pharmaceuticals (20). In contrast to the YES, the E-SCREEN provides information on cellular effects, i.e. how will a compound in isolation or a subjected sample affect cells with an intricate number of signal transduction pathways.

In conclusion, several biological methods exist to monitor pollution. However there is a global trend to move away from general pollution to determining what type of pollution is present. Two bioassays used for detecting pollution were discussed and their differences highlighted. However, these bioassays require more time to prepare, can be expensive (e.g. the E-SCREEN) and their pollution information are still not exact. However, analytical chemistry methods exist that can determine exactly what type of polluting chemicals are present and from these conclusions can be drawn on how they might affect things like the endocrine system of mammals when present in for e.g. water resources. These methods will be discussed in the next section.

2.4 Analytical chemistry methods employed to monitor pollution

2.4.1 Analytical chemistry methods overview

Globally there is effort to develop methods that will enable exact determination of the type of pollutant present in water. However, due to the large number of possible pollutants, method development proves a daunting task. Moreover, detecting some pollutants at low levels further stonewalls method development. Nevertheless, existing chromatographic techniques, such as ultra-performance liquid chromatography (UPLC), gas chromatography (GC) and super-critical fluid chromatography (SFC) coupled to mass spectrometry instrumentation, such as Time-of-Flight (TOF), quadrupoles (Q) and ion-traps, prove to be the future for identifying pollution. These methods are sensitive and can identify many pollutants simultaneously with high precision. Figure 2.3 illustrates how these systems are able to determine the exact chemical pollutants in a sample simultaneously.

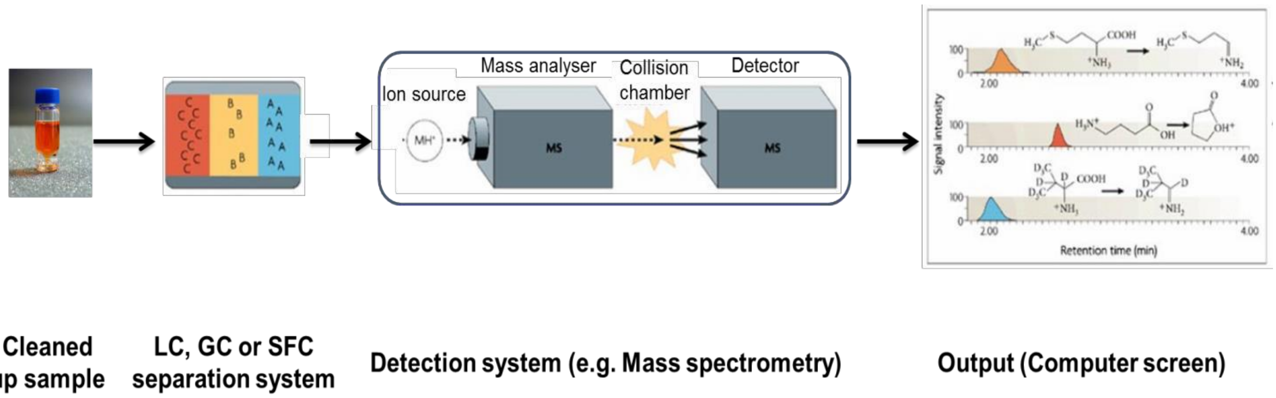

Figure 2.3 Graphic illustration of how a pollutant can be identified from a sample using analytical chemistry techniques. Here the chromatographic system is connected to a mass spectrometer. The cleaned up sample refers to a sample that underwent pre-clean up to remove debris and any unwanted matrix constituents such as salts and metals. The output generated on the computer screen is usually generated with a software package such as Masslynx

Sections 2.4.2, 2.4.3 and 2.4.4 will deal with the above mentioned chromatographic systems and the different detector systems that have been coupled to them. However, special regard will be given to mass spectrometry (section 2.4.5) as a detector system, as its use is becoming ever more important and the chromatographic systems used in chapter 3 all use this technology. The second last section (section 2.4.6) will deal with the chemical process of derivatization as a method to enhance GC-, LC- and SFC-MS analysis, while the last section (section 2.4.7) will take everything together and put things in perspective. Together these sections aim to provide background information and a deeper understanding and knowledge into the systems used in chapter 3.

2.4.2 Gas chromatography

Although chromatography dates back to 1903, modern gas chromatography as it is known today was only first developed and published by A.T James and A.J.P Martin in 1952 (21). In their publication (22) the authors described the modifications necessary to allow the compression of the mobile phase to the theory of gas-liquid partition chromatography (GLPC), or GC, originally proposed by Martin and R.L.M Synge in 1941. Martin and Synge‗s theory stated the following: ‗a very refined separation of volatile substances should be possible in a column in which permanent gas is made to flow over a gel impregnated with a non-volatile solvent' (23). From these two publications followed the concept of separating volatile substances on a column with a gaseous mobile phase.

Today these theories and their improvements are practically invisible with state-of-the-art equipment implementing almost a century worth of knowledge in mere minutes. Currently, the most noteworthy improvement to conventional GC is the use of a pair of columns, normally one polar and the other non-polar, in what is known as two dimensional GC, or GCxGC chromatography. In this technique separated compounds from the first column is transferred to a second column for further separation. Finally, specialized software renders the chromatograms of both columns in two dimensions (24). The advantage of using this technique is that compounds with similar boiling points co-eluting on one column can be separated on the second before traveling to the detector, yielding improved results.

To detect the volatile compounds separated by GC and GCxGC various detection methods have been employed over the years such as thermal conductivity- (TCDs), flame ionization- (FIDs), catalytic combustion- (CCDs), discharge ionization- (DIDs), electron capture- (ECDs), helium ionization- (HIDs), nitrogen-phosphorus- (NPDs), infrared- (IRDs), photo-ionization- (PIDs), pulsed discharged ionization- (PDDs) and vacuum ultraviolet detectors (VUV) as well as mass spectrometers (MS). Of these, TCDs and FIDs are the most commonly used detectors for GC (21, 25, 26). However, the use of MS is becoming ever more increasing as smaller quantities of sample are needed and these detectors yield improved sensitivity. The use of MS as a detector will be discussed in section 2.4.5.

2.4.3 Liquid chromatography

Although the ground breaking work on liquid chromatography (LC) by Mikhael Tsvet should not be forgotten, Martin and Synge's work also set the stage for modern liquid chromatography with the development of high performance liquid chromatography (HPLC) in the early 1960s and 1970s. Seeing that GC relies on the volatility of compounds, scientists in the field of biology found it difficult to separate biological molecules with GC as these normally had too high a molecular weight or were far to polar to be volatile enough for use in GC instrumentation. With this came the development of HPLC as a consequence of both the unsuitability of GC and the long separation times of existing LC techniques.

Unlike conventional LC, HPLC entails the separation of compounds in a sample on a column under highly pressurized liquids. Samples injected on these instruments do not require volatilization of compounds for analysis and have the added benefit of shorter separation times (27). With the break of the 21st century advances in column technology and instrumentation ‗upgraded' HPLC to UPLC and with that again came significant increases in speed, sensitivity, resolution and shorter separation times (28).

Like GC, a number of LC detectors have also been used over the years. These detectors include the likes of electrochemical-, refractive index-, UV-, fluorescence-, light scattering-, conductivity, infrared, chemiluminescent-, chiral, corona discharge-, radio-active- and MS detectors (29). Here, the most widely used detector is probably the UV/VIS detectors coupled to HPLC, while modern UPLCs are mostly coupled to photo diode arrays (PDA, UV), fluorescent or MS detectors, or a combination thereof, but are not limited to only these.

2.4.4 Super-critical fluid chromatography

Much effort was put into the development of GC and LC over the past century to the point of modern day instruments, with improvements initially focussed on GC and later HPLC. However, it was probably only in the past three decades that improvements to SFC let to the recognition of the technique as a useful separations method. This is particularly concerning as the first report of SFC was in 1962 by Klesper and colleagues, round about the same time HPLC was the technology of interest. In SFC, a gas at its super-critical point is used as the mobile phase to separate compounds in a sample injected on a column similar to that of HPLC/UPLC. Like HPLC, a major limitation of SFC was due to instrumentation that was unable to handle the pressures required. However, the improvement of HPLC equipment lead to the improvement of SFC and consequently improved performance. While GC had a high resolution and sensitivity, HPLC and later UPLC had shorter sample run times. Importantly, GC often required compounds to be derivatized (e.g. estradiol) – a labour intensive and expensive task, to increase the compound‘s volatility, while, HPLC had a lower resolving power than GC (30).

SFC, on the other hand, has properties of both GC and HPLC/UPLC. Gas compressed to a super-critical fluid has a density similar to that of a liquid in HPLC/UPLC, gas-like viscosity similar to that of GC, while at the same time having a diffusivity somewhere between that of GC and HPLC/UPLC. This makes SFC ideal in that the technique could have high resolution, sensitivity and shorter sample run times (30). Indeed, in 2012 the Waters Corporation released the first of its kind SFC instrument, called the UPC^2, able to fulfil the requirements of high resolution, sensitivity and shorter run times. In addition, the UPC^2 also provides high selectivity for compounds where it is able to separate structural analogues, enantiomers, diastereomers and isomers. According to Waters, the UPC^2 is further both economically and environmentally friendly, making it an attractive alternative to GC and HPLC/UPLC (31). The efficiency of this system was recently shown in two articles focussing on the separation of endogenous hormones normally not possible or economically viable on GC or HPLC/UPLC (32, 33).

Since its first appearance in 1962, SFC instrumentation has been coupled to both detectors normally associated with GC and LC. These include FIDs, UV photometers, PDAs, and MS detectors, with the most frequently used being FIDs from the ‗60s to the ‗90s and MS detectors from the 21st century (30).

2.4.5 Mass spectrometry as an ultra-senstive detector system for GC, LC and SFC

The ion source

After chromatographic separation, compounds travel to the MS unit where they are first ionized by the ion source to form precursor ions. For vapours and gasses, such as those used for the mobile phase of GC instruments, electron- or chemical ionization (EI or CI) are used, while electrospray- and matrix assisted laser desorption ionization (ESI or

MALDI) are used for liquid mobile phases such as those from UPLC instrumentation. Of the aforementioned ionization processes CI, ESI and MALDI are soft ionization techniques, while EI is a hard ionization technique – referring to the ability of an ionizing source to cause maximal fragmentation compared to minimal fragmentation in soft ionization. GC is most often associated with hard ionization as the high degree of fragmentation provides unique insight into the structural integrity of a molecule that allows the identification of unknown compounds from libraries. In contrast, LC is more commonly associated with soft ionization with minimal fragmentation, allowing the determination of e.g. molecular weights of biological molecules (34).

The mass analyser

Following ionization, the ions are transported by electric or magnetic fields to the second component of a MS unit – the mass analyser. Here, they are separated according to their mass-to-charge ratio. This step is then normally followed by a collision step, with a specific collision energy (CE), in the collision chamber to generate a wide variety of product ions. The three most commonly used mass analysers are time-of-flight (TOF), quadrupole and ion trap analysers. TOF analysers operate on the time it takes an ion to travel to the detector when accelerated by an electric field. In contrast, quadrupoles selectively filter ions from an electric field, using radio frequencies and direct current voltages, according to their m/z ratio before reaching the detector (35–37). Both analysers offer great advantages to the user. Quadrupoles offer selectivity, especially when used in tandem (MS/MS), for targeted method approaches such as looking for a specific drug in a water matrix. The downfall of the targeted approach is that the same drug modified, e.g. by chlorination through waste water treatment, in the same water matrix might be missed. TOFs, on the other hand, offer the screening of a sample for ions with masses over a specific range and therefore can detect e.g. both the ion in question and modified forms. However, TOF lacks sensitivity and linearity when compared to quadrupoles and therefore are rarely used for quantitation. In contrast to TOFs and quadrupoles, ion traps store selected ions in a trap where they can be manipulated before reaching the detector (38). This makes ion traps highly sensitive and provides high resolution MS. However, the trapping of ions comes at a price : the quality of the mass spectrum can be influenced by other ions generated in the experiment that is also trapped by the ion trap analyser (39).

The detector

Finally, the MS detector measures the current produced when a product ion passes through an electromagnetic field or the charge induced when it hits a metal plate. It is then also this transition detected from the precursor ion (the ion produced by the ion source and monitored with the mass analyser) to a product ion (the ion generated from collision) that is used in single or multiple reactions monitoring (SRM or MRM). The resulting transition is specific to each compound and therefore is the identity of the compound of interest. The type of detector used in a MS depends on the analytical application and instrument design. A number of detectors have been used over the years. These include photographic plate-, Faraday cup-, electron multiplier (EM)-, electro-optical ion-, orbitrap- and Fourier transform ion cyclotron resonance (FTICR) detectors, with the most frequent detector used today being the EM (40).

2.4.6 Derivatization as a tool for enhancing GC-, LC and SFC-MS analysis

Improvements to chromatographic techniques or their detectors can significantly improve obtainable results. However, results can also be improved through alternative methods. One such a method is derivatization. Plainly, derivitization is a chemical process in which a compound is modified by the addition of a derivitizing agent to a functional group of the compound, forming a product similar to the original with altered physiochemical properties. The altered properties can then be used as an advantage in analytical techniques. One of the best known groups of chemicals often derivatized for analysis is the estrogens and their synthetic analogues. Analysis with GC-MS proves difficult as these compounds have too low a volatility, while difficulty in their ionization is the primary concern when using LC-MS. However, the problem with low volatility and insufficient ionization can easily be solved by derivatizing these compounds with agents such as Dansyl Chloride (DNCl, LC-MS), or *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, GC-MS). For a detailed summary of currently available derivatization techniques aimed at targeting the ketone and aldehyde, alcohol and phenol, carboxylic acid, amine and thiol functional groups, the reader is kindly referred to the review by Bao-Ling Qi *et al.* (41) (LC-MS) and a chapter by Francis Orata (42) (GC-MS).

2.4.7 Connecting the dots of the chromatographic systems with those of the mass spectrometry systems and derivatization

From sections 2.4.2, 2.4.3, 2.4.4 and 2.4.5 it is evident that a number of analytical systems are available to determine exactly what type of pollutants are present in a sample and that mass spectrometry provide a high sensitivity for the different chromatographic systems.

Furthermore, together with derivatization even difficult-to-analyse pollutants can be detected in samples. Therefore, the use of chromatography coupled to mass spectrometry is highly recommended for pollutant detection. However, unlike the biological methods, methods developed on the analytical instrumentation for detection of pollutants in samples require each method to be validated. This process can be an expensive and tedious process. Nevertheless, after method validation, analyses of samples become more affordable. For a detailed overview on chromatography mass spectrometry method validation the reader is referred to two articles by Kruve *et al.* (43, 44).

2.5 The risk of EDC pollutants on humans and animals using breast cancer as an example of a possible consequence

2.5.1 Overview

In section 2.2 the global state of water scarcity and the risk of polluting the remaining water resources were discussed, while sections 2.3 and 2.4 focussed on how to monitor pollution with biological and analytical chemistry methods. This section, in contrast, aims to emphasize the risk to humans and animals if some of the discussed pollutants are detected in water resources. The discussed pollutants all belong to the group known as EDCs. The focus will fall on these compounds as they have a tendency to lead to bio-magnification with health consequences throughout the different trophic levels. As humans are on top of the food chain, the discussions to follow will mostly deal with human physiology and the consequences related to us. To conceptualize the incredible risk that these compounds pose in our water resources, BC is used as one of the many possible consequences. The three sections (Sections 2.5.2, 2.5.3 and 2.5.4) to follow will deal with the human endocrine system and its function in the regulation of homeostasis. The remaining sections will focus on ED with specific regard to the routes of EDC exposure, EDC modes of action, BC and the most common EDC and their role in BC.

2.5.2 Maintaining homeostasis

Levels of organisation in the human body

The human body can be organised into five levels – chemical, cellular, tissue, organ and whole-body system level. The chemical level our bodies are made up of is a combination of trillions of atoms. These atoms essentially form the molecules of life, such as carbohydrates, fats, proteins and nucleic acids. Organising these molecules into the most basic units of life we find cells. Cells carry out the processes of life, such as obtaining nutrients and oxygen from its environment, eliminating by-products and waste formed during chemical reactions and synthesizing the components needed for continued growth and survival. Grouping cells of similar specialization we find tissue. Each of the four primary tissue types – muscle, nervous, epithelial and connective, contain an intricate number of subtypes that each fulfil a particular objective. An example of this is muscle tissue. Muscle tissues can move the skeleton – termed skeletal muscles, pump blood from the heart to the rest of the body – termed cardiac muscles, and move contents through hollow tubes and organs – termed smooth muscles. Together, the different types of tissues make up an organ. Organs each perform a particular function (e.g. O_2 and CO_2 exchange by the lungs) or functions (e.g. food storage and digestion by the stomach) that enable the body system to accomplish a common activity (e.g. respiration by the respiratory system) that is essential for survival of an organism.

As can be seen each level of organisation contributes to both the levels above and beneath them, and that no level can exist without the levels beneath it. Together these organisational levels work together to maintain the phenomena known as homeostasis. Defined as a dynamic steady state, homeostasis is essential for the survival of each cell, so that each cell, through its specialized activities, contributes as part of a body system, to the maintenance of the internal environment shared by all cells (45) (Figure 2.4).

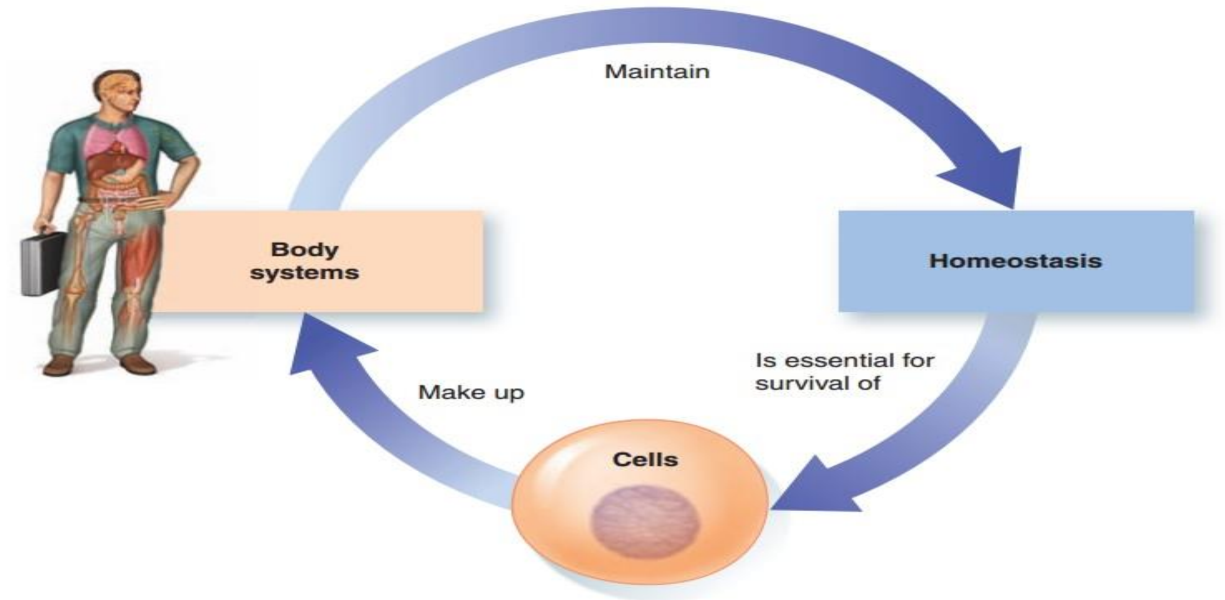

Figure 2.4 Interdependent relationship of cells, body systems, and homeostasis (45). The one system cannot function without the other. Together cells maintain body systems and vice versa.

Homeostasis is maintained by 11 body systems working together

Whether it be relaying information (nervous system) to muscle tissue in order to prevent the burning of your hand or the physical activity of moving the hand (muscular system) attached to the rest of the body (skeletal system); be it the lungs (respiratory system) supplying oxygen, via the blood (circulatory system), and therefore electron acceptors used during the metabolism of food (digestive system) for fighting off or protecting against pathogens (immune and integumentary system); or the removal of unwanted waste/by-products (urinary and digestive system) from food and cells; or even the supply of hormones driving reproduction (endocrine and reproductive system); all contribute to homeostasis.

2.5.3 The human endocrine system

Background

Of these 11 body systems, the nervous system and endocrine system (all hormone-secreting glands) regulate the majority of bodily actions. However, whereas the nervous system‘s main function is to regulate activities at great speed e.g. reflex reactions, the endocrine system regulates activities that necessitate duration such as fight or flight. Some of the endocrines major functions are the regulation of nutrients, maintenance of electrolyte concentration in the extra cellular fluid, to help the body cope with stress by inducing adaptive changes, monitoring red blood cell production and regulating reproduction. Unlike the nervous system that transmit electrical signals to the brain for interpretation and back to the relevant organ for effect, the endocrine system secretes hormones into the blood for transport to its target cell or organ, where they coordinate their effect.

There are some 50 odd hormones, not including their chemical intermediates, secreted and circulating in the human body, each with its own function and target cell or organ. These hormones are biochemically classified as peptide, amine, steroid or thyroid hormones. Peptide and amine hormones are hydrophilic in nature, whereas steroid and thyroid hormones are lipophilic. Of these four biochemical classes, peptide and steroid hormones are the most prevalent hormones. However, the discussions to follow will mainly deal with steroid and thyroid hormones as their deregulation can affect an organism adversely.

Regulation of the endocrine system by the HPG, HPA and HPT axes

The secretion of effector hormones, such as androgens, estrogens, progestogens, thyroxine (T_4), aldosterone and cortisol, are mainly controlled by three pathways – the Hypothalmic-Pituitary-gonadal axis (HPG), Hypothalmic-Pituitary-Adrenal axis (HPA) and Hypothalmic-Pituitary-Thyroid axis (HPT) axis (Figure 2.5).

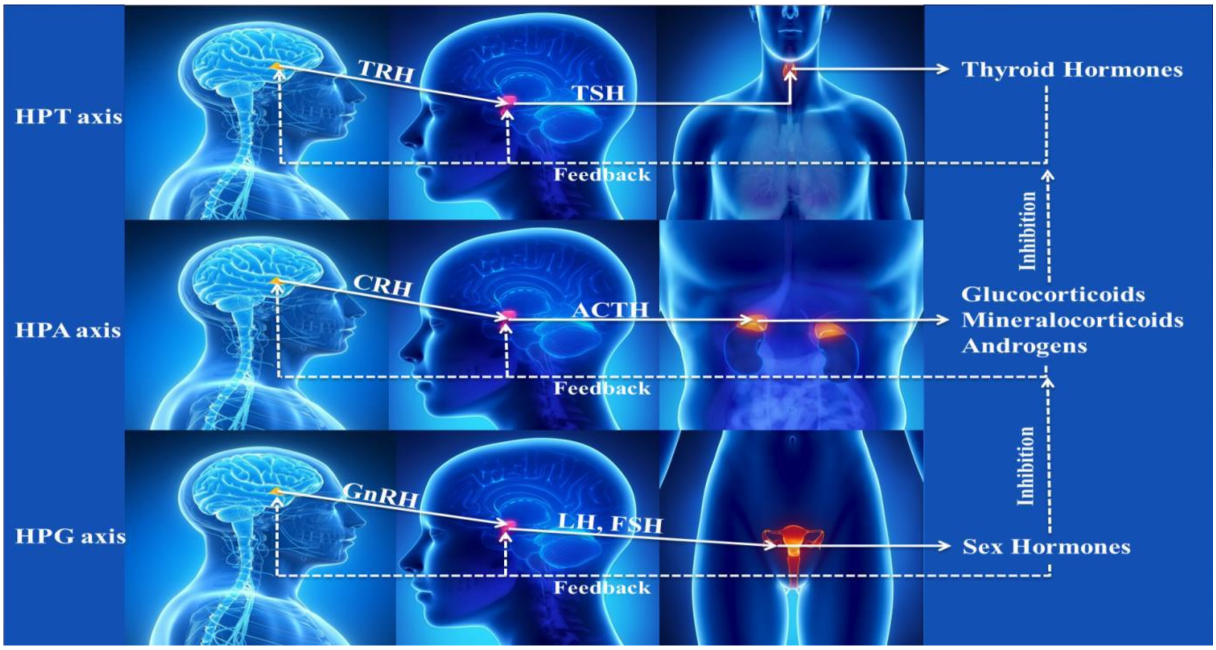

Figure 2.5 The three hypothalamus controlled axis involved in maintaining endocrine homeostasis. TRH, Thyroid-releasing hormone; TSH, Thyroid-stimulating hormone; CRH, Corticosteroid-releasing hormone; ACTH, Adrenocorticotropic hormone; GnRH, Gonadotropin-releasing hormone.

The HPT axis is responsible for the regulation of metabolism and growth and the HPA for stress, digestion, immunity, mood etc., while the HPG axis mainly play a role in reproduction and life cycles. In the HPT axis, the hypothalamus regulates the levels of T_4 and tri-iodothyronine (T_3) by responding to low or high levels of T_3 and T_4 in the blood. When either T_3 or T_4 is low, the hypothalamus releases thyroid-releasing hormone (TRH) which stimulates the release of thyroid-stimulating hormone (TSH). TSH in turn, acts on the thyroid to produce T_3 or T_4, returning the levels in the blood to normal. Similarly, the HPA and HPG axis respond to low levels of glucocorticoids, mineralocorticoids and sex hormones by releasing corticosteroid-releasing hormone (CRH) or gonadotropin-releasing hormone (GnRH) followed by the production of adenocorticotropic hormone (ACTH), luiteinizing hormone (LH) or follicle-stimulating hormone (FSH), before the adrenal (HPA) or gonadal (HPG) hormone levels in the blood return to normal. However, in the case of excess sex steroids, glucocorticoids, mineralocorticoids or thyroid hormones, these hormones suppress the release of their stimulating or releasing hormones through negative feedback loops. Finally, the three axes are linked in that they can coordinate each other‘s outcome through product inhibition. Figure 2.5 only illustrates the basic regulation of the endocrine system by the three axes but is in fact a complex integrated system regulated by many mediators. Disruption of the endocrine system can therefore have serious consequences to an organism.

2.5.4 Signal transduction

Signal transduction is a complex process (Figure 2.6) wherein extracellular signals, in the form of chemical messengers bring about an intracellular response such as the activation of genes responsible for cell proliferation. These chemical messengers include hormones, pheromones, neurotransmitters and growth factors, cytokines and cell adhesion molecules. Typically these messengers are synthesized by specialized cells such as those from the endocrine organs – the testes, ovaries and adrenal cortex. After release into e.g. the blood stream, these molecules are transported to the relevant tissues before they are detected by intracellular receptors or on the surface of a cell in tissues. Consequently, a response is initiated and where-after the molecules are removed again. The extracellular receptors detecting these molecules include G-protein coupled receptors (GPCRs), enzyme-linked receptors [receptor tyrosine kinases (RTKs), receptor tyrosine phosphatases, serine/threonine kinases, receptor guanylyl cyclases and histidine kinases], integrins, toll-like receptors (TLRs) and ligand-gated ion channels. Alternatively, the intracellular receptors include nuclear and cytoplasmic receptors. For the purpose of this thesis and content to follow only signal transduction through GPCRs, RTKs and nuclear receptors will be of value.

Nuclear receptor mediated signal transduction

Nuclear receptors (NRs) are a group of steroid hormone sensing receptors. However, these receptors are not limited to only steroid hormones and are known for their ability to also bind xenobiotics – chemicals not native to an organism. Forty-nine, 48 and 47 NRs have been identified in mice, humans and rodent respectively (47). These receptors can be categorized according to their sequence homology into the subfamilies thyroid hormone receptor-like, retinoid X receptor-like, estrogen receptor-like, nerve growth factor IB-like, steroidogenic factor-like, germ cell nuclear factor-like and miscellaneous (48, 49). For the purpose of the section on endocrine disruptor modes of action, only the subfamilies thyroid hormone receptor-like, retinoid X receptor-like and estrogen receptor-like are of importance and will be discussed throughout this thesis. However, the main focus will be on nuclear receptors.

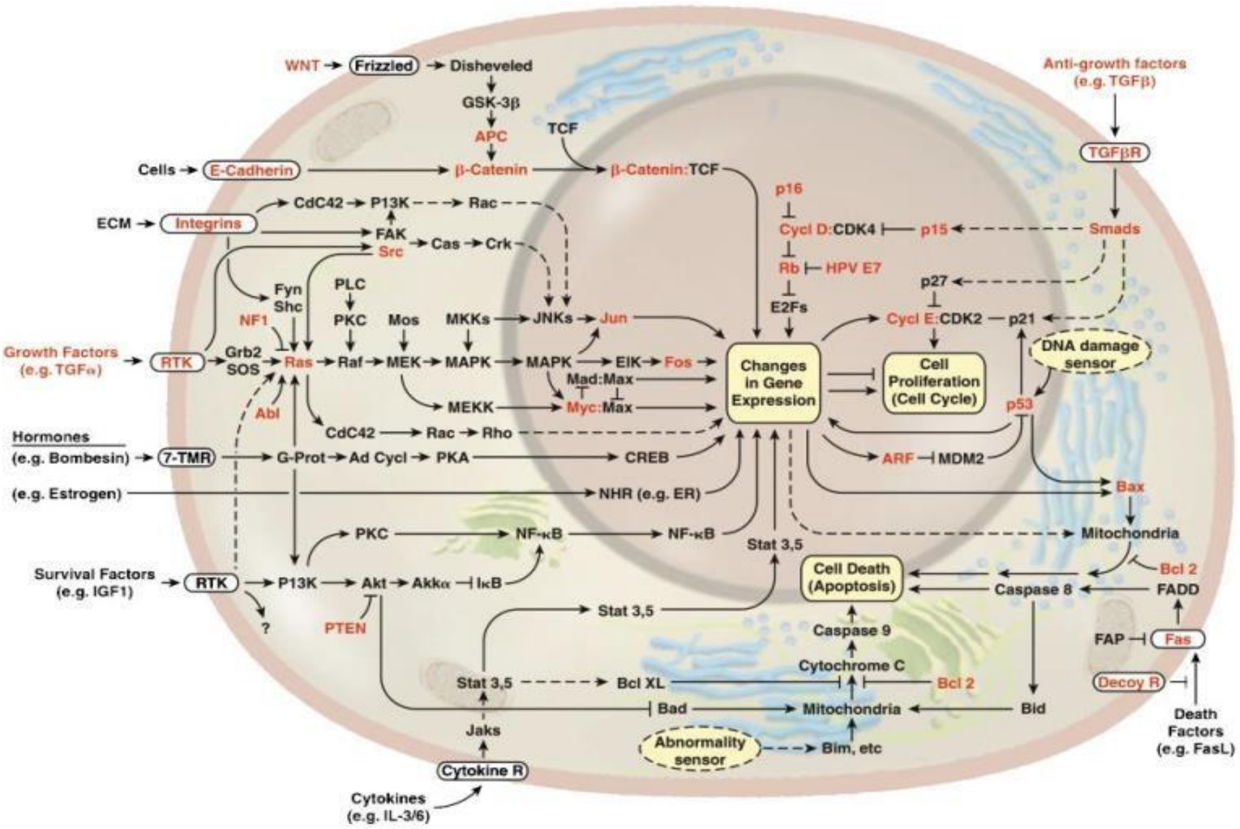

Figure 2.6 Complexity of signal transduction in mammals. Adapted from Hanahan and Weinberg (46).

Nuclear receptors can be subdivided into four mechanistic classes namely Type I, II, II and IV (50, 51). This thesis will focus only Type I and II mechanism (Figure 2.7 and Figure 2.8). Type I receptors (Figure 2.7) are activated by steroid hormones/xenobiotics after crossing the cell membrane and binding to the nuclear receptor/heat shock proteins (HSPs) complex (52). This causes the HSPs to dissociate and homodimerize, followed by translocation to the nucleus where the dimerized complex binds the hormone response element (HRE), recruits co-activators and target gene transcription is initiated (52). This is known as transactivation. In contrast, transrepression involves the binding of a single liganded-receptor and NFκB/AP-1 to NFκB/AP-1 gene element (not shown in Figure 2.7), followed by repression of the target site genes. Type 1 receptors include the androgen receptor (AR), estrogen receptors (ERs), glucocorticoid receptors (GRs) and progesterone receptors (PRs) (53).

Type II receptors (Figure 2.8) reside within the nucleus of a cell regardless of ligand binding status (52). Unlike type I receptors, type II receptors heterodimerized with another NRs. The most prominent of these being the retinoid X receptor (RXR) (52). Upon ligand binding to NR, corepressors dissociate and co-activators proteins recruited, where-after additional proteins such as RNA polymerase is finally recruited for transcription of the target genes (52). Type II receptors include RXR, PXR, TR and CAR (52, 54).

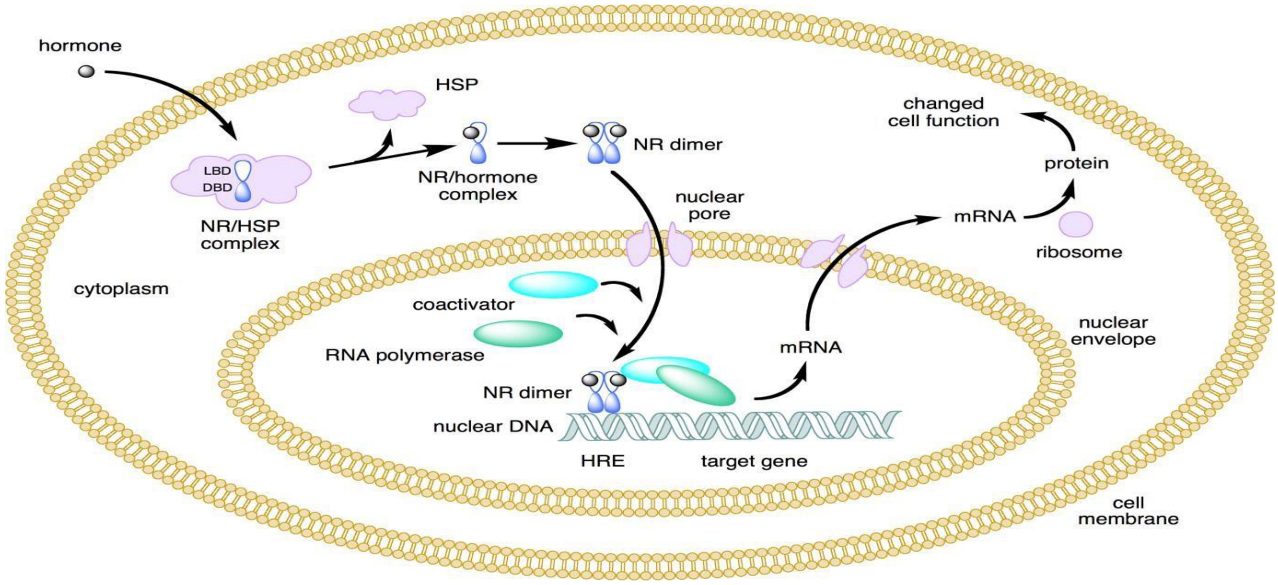

Figure 2.7 Signal transduction mediated through class I nuclear receptors. NR, Nuclear receptor; HSP, Heat shock protein; HRE, Hormone receptor element.

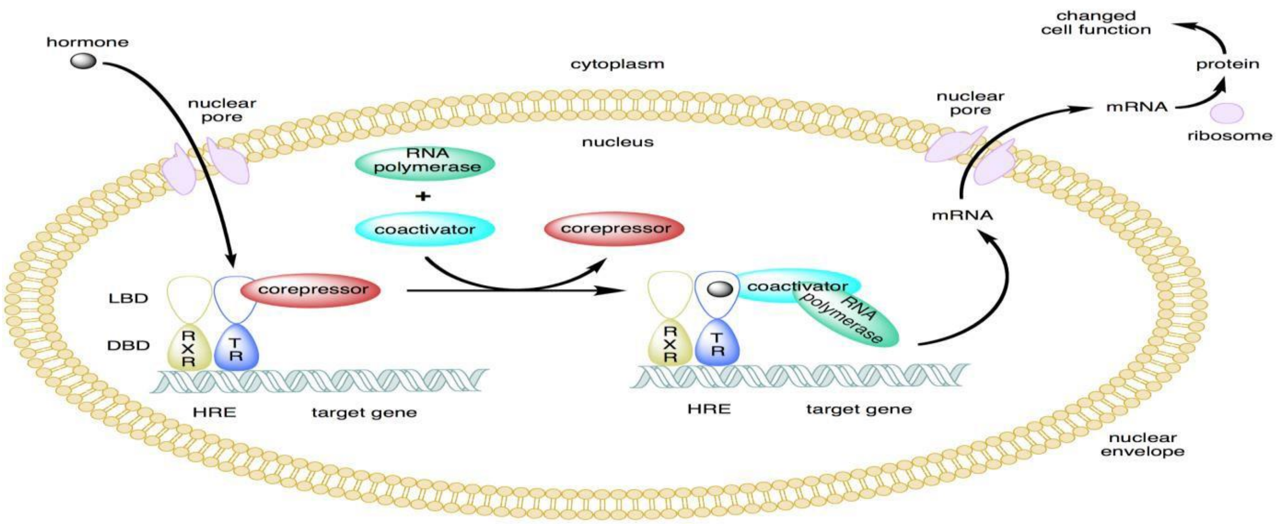

Figure 2.8 Signal transduction mediated through class II nuclear receptors. LBD, Ligand binding domain; DBD, DNA binding domain; HRE, Hormone response element; RXR, Retinoid X receptor; TR, Thyroid receptor.

GPCR and RTK mediated signal transduction

GPCRs and RTKs are part of a superfamily of transmembrane receptors, the other being ligand-gated ion channels, that bind ligands on the surface of the cell (Figure 2.9) (55). GPCRs are solely expressed on the membranes of eukaryotes and are activated by pheromones, neurotransmitters, hormones, odours and photosensitive compounds (55–57). The two principal GPCR activated pathways include the cyclic AMP (cAMP) and phosphatidylinositol (PI) pathway (58). In contrast, RTKs bind majorly growth factors, cytokines and hormones such as epidermal growth factor, hepatocyte growth factor, insulin and nerve growth factor (55, 57). These receptors play an integral role in normal cellular processes but have been linked to the development and progression of cancer (59).

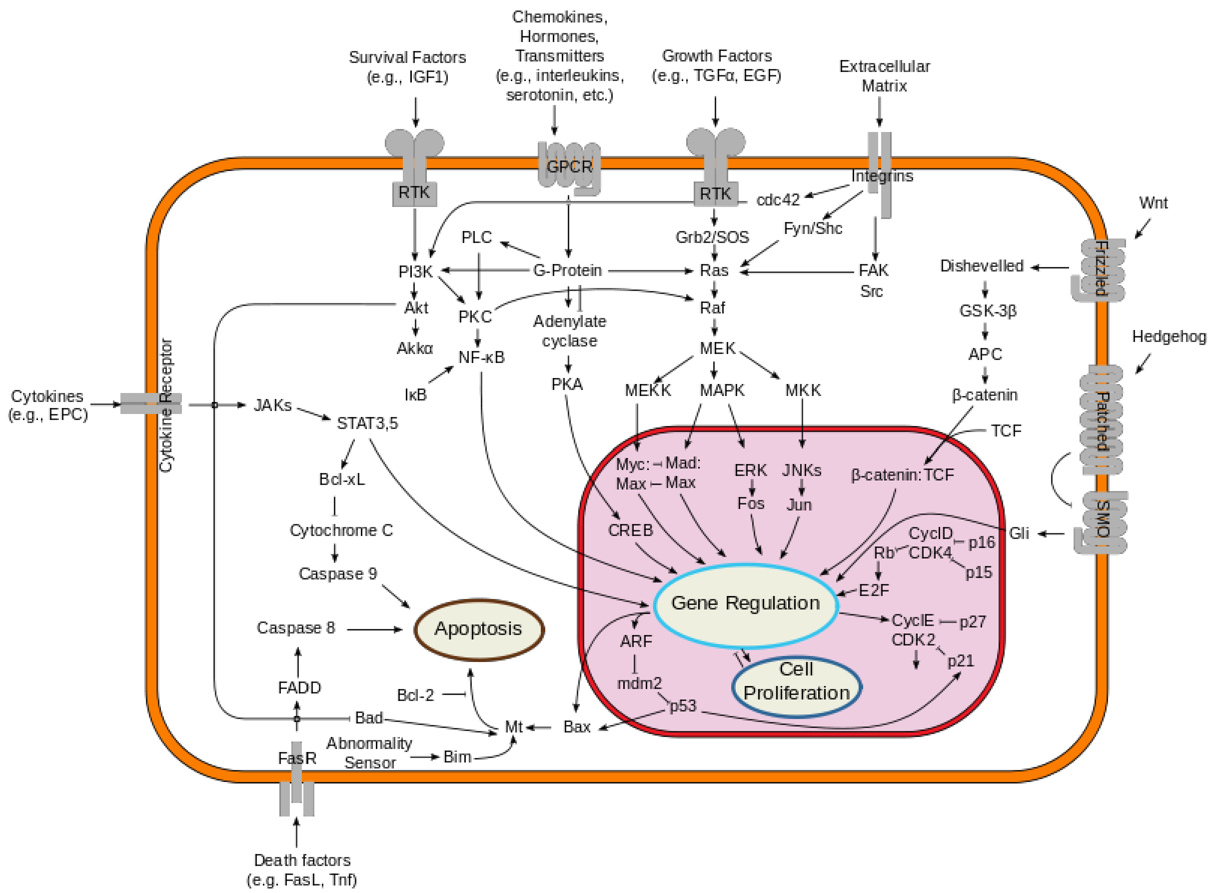

Figure 2.9 Simplified signal transduction pathways through cell surface receptors.

Upon activation of a GPCR, the receptor undergoes a conformational change that activates a G-protein, transducing the signal to effector enzymes such as adenylyl cyclase and phospholipase C. These effector enzymes then produce second messengers such as cAMP, inositol-1,4,5-triphosphate (IP3) or diacylglycerol (DAG) that can stimulate the release of Ca^{2+} from the endoplasmic reticulum or the activation of protein kinase C (PKC) to initiate a phosphorylation cascade leading to a cellular response. Alternatively, Ca^{2+} released from endoplasmic reticulum can also stimulate PKC to cause the phosphorylation of proteins as the case was with DAG (55, 57).

In contrast to GPCRs, RTKs dimerize upon ligand activation. This enables the cytoplasmic structures of each monomer to *trans*-phosphorylate the other. Phosphorylation of specific tyrosine residues on the activated receptor complex causes the binding of Src homology 2 domains – conserved protein domains important in signal transduction, and phosphotyrosine binding domains. Consequently, the RTK transmit a hormone signal to a GTPase switch protein, Ras, which initiates a kinase signalling cascade through the serine/threonine-specific kinases, Raf and MEK, to mitogen-activated protein kinase (MAPK) cascade, evidently leading to a cellular response such as cell proliferation (55, 57).

EDCs initiate signal transduction leading to uncontrolled cellular processes

Cells in the human body constantly respond to stimuli from either the nervous system or endocrine system, which enables them to perform their normal physiological functions. The HPA, HPT and HPG axes ensures that cells maintain homeostasis throughout these routine cycles by constantly adjusting the concentration of effector hormones. These effector hormones mediate their effect through the signal transduction pathways discussed above and each effector hormone controls a specific onset of cellular circumstances – whether it is cell proliferation or senescence, apoptosis or necrosis, metabolism or gene expression. The emphasis however is on controlled regulation, but every so often cellular processes go awry leading to things like cancer. The circumstances leading to this onset can be multifold but organizations such as WCRFI attributes much of what is known about the initiation and progression of cancer to our lifestyles.

It is believed that our continuous exposure to the diverse set of chemicals on a daily basis contributes to cancer. Indeed, EDCs such as the ones discussed in the next section have been researched extensively and many linked to the disruption of the endocrine system. These EDCs are shown to interfere with the endocrine system mediate responses such as cellular proliferation through signal transduction pathways disrupting normal controlled cellular responses and processes. To substantiate this view the next section will deal with the endocrine disruptor, the endocrine disruption phenomenon, routes of exposure, modes of action and their link to breast cancer initiation, progression and recurrence.

2.5.5 Breast cancer is one of many possible consequences of ED

EDCs

According to the National Institute of Health (NIH), endocrine disruptors are ‗chemicals that may interfere with the body‘s endocrine system and produce adverse developmental, reproductive, neurological and immune effects in both humans and wildlife‘. Although correct, this definition only describes interference with the body‘s endocrine system and the adverse side-effects and makes no effort to point out the possible modes of action through which these chemicals interfere with the endocrine system to bring about the said side-effects. A more comprehensive definition is probably that of Crisp and associates on behalf of the United States Environmental Protection Agency (USEPA). In their 1998 report USEPA defines an endocrine disrupting chemical (EDC) as an ‗exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development and behaviour‘. This is evident from numerous studies showing that EDCs can alter the signalling and function of endogenous hormones (60–62), alter the biosynthesis of steroid hormones (63), cause teratogenesis (64), disrupt ovarian function (65, 66), reduce fecundity (61, 67), influence the timing and onset of menarche (61, 68), lead to early menopause (61), cause irregular lactation (69) and cancer (70–72). In addition, EDCs influencing steroid-sensitive tissue was found to disrupt the function of the CNS (73–75), lipid homeostasis (76–78), immune system (79, 80), glucose levels (81, 82), thyroid function as well as act as epigenetic modulators (83, 84).

In 1989, Barker and colleagues postulated that environmental influences early in life lay the foundation for adult disease (85). This became evident in a study by Guillette *et al.* (86) showing the feminization of juvenile American alligators in Lake Apopka exposed to contaminated water. Sumpter and Johnson (87) noted that environmental endocrine disruption is caused by co-exposure of EDC at low concentrations, leading to additive and synergistic effects. With an estimated 10 000 EDCs among common daily exposures (68), disease programmed at an early age is therefore inevitable. In addition, most EDCs perform their modulatory effect at concentrations well below the no-observed-effect-level (NOEL) (88), thereby limiting our ability to link certain EDCs to specific diseases and therefore attempts at prevention. With this in mind, it is no wonder that the United States Federal Interagency Breast Cancer and Environmental Research Coordinating Committee (IBCERCC) in 2013 stressed the necessity to study the effect of chemical exposure, predominantly EDC exposure, on a disease such as breast cancer (89).

Routes of EDC exposure to humans and animals

The routes of EDC exposure to humans are practically limitless in our fast living, large production scale, processed, high performance, wealthy lives that we live, or try to live. As a consequence both humans and animals will suffer a similar fate – diseased-state lives. Unlike humans, animals don‘t have a choice of healthy living and therefore the onus lies with us humans to take care of the living that keeps us alive. With an estimated 1.3 billion tonnes of solid waste generated annually by cities around the world, leaching of EDC from plastics, industrial waste and medical waste etc. into our environment, especially into our water resources, are unavoidable (90). Moreover, every year an estimated 330 km^3 of municipal waste water is generated (91). This generated waste water is high in soaps, emulsifiers, cleaning products, pharmaceuticals and personal care products (PPCPs), many of which has been linked to some sort of ED in both humans and animals.

Although waste water treatment is generally considered complete, a number of studies show the opposite, with certain chemicals, such as Estrone (E_1), 17β-Estradiol (E_2), 17α-Ethinyl estradiol (EE2) – synthetic estrogen used in contraception, Carbamazepine – an antiepileptic, Venlafaxine – an antidepressant, Tramadol and Methadone – analgesics, Ketamine – an anaesthetic, Tamoxifen (TAM) – an anticancer drug, partially or incompletely removed by treatment plants (92, 93). These chemicals then end up in the environment where they can elicit an effect such as ED. The effect of chemicals such as Carbamazepine (CBZ), triclosan (TCS) and Diclofenac on aquatic life has been documented by Nassef *et al.* (94) and Jarvis *et al.* (95), the effect of PPCPs on aquatic life in a review by Brausch and Rand (96) and a case study by Zenobio *et al.* (97), while Jobling *et al.*(98, 99), Knacker *et al.* (100), Morthorst *et al.* (101) and Skolness *et al.* (102) showed ED in fish, Carey and Bryant (103), van Wyk *et al.* (104), Hecker *et al.* (105) and Boone *et al.* (106) ED in amphibians, Kelce *et al.* (107), De Angelis *et al.* (108) and Rider *et al.* (109) ED in rodents and Prahalada *et al.* (110) ED in primates, associated with contaminated water sources.

There is also a growing concern that effluent water or biosolids originating from waste water treatment plants that is used for irrigation and fertilization may further contribute to EDC build-up in the food chain by accumulating in the ground and evidently plants. This concern was probably initiated first by a review published by Xia *et al.* (111), in which the authors discussed the possibility of certain PPCPs to leach from biosolids into biosolid-applied soils. Subsequent work by the likes of Wu et al. (112), Karjanapiboonwong et al. (113), Wu et al. (114), Wu et al. (115), Dodgen et al. (116) and Dodgen et al. (117), described the uptake of chemicals such as CBZ, diphenhydramine, triclocarban (TCC) and TCS, diazepam, naproxen, diclofenac, bisphenol A (BPA), 4-Nonylphenol and EE_2, to name only a few, in roots and shoots of plants such as pepper (*Capsicum annuum*), tomato (*Lycopersicon esculentum*), collard (*Brassica oleracea*), lettuce (*Lactuca sativa*), radish (*Raphanus sativus*), spinach (*Spinacia oleracea*), Pickling cucumber (*Cucumis sativus*), Soybean (*Glycine max*) and Pinto bean (*P. vulgaris*), validating the concern that PPCPs – many of which are known EDCs, are possible to accumulate in agricultural plants.

However few, EDC exposure through air has been reviewed by Rudel and Perovich (118). In their 2009 review, the authors highlights how six semi-volatile chemical classes – polychlorinated biphenyls (PCBs), brominated flame retardants (PBDEs), pesticides, phthalates, alkylphenols and parabens persist in indoor and outdoor air, can have potential health effects.

In summary (Figure 2.10), anthropogenic EDC originate from a number of point sources such as solid waste at landfills that leach into the environment, waste water treatment plant (WWTP) effluent that end up in fresh water resources and agriculture as well as the air. These transmission sources then provide the routes for entry into humans and animals. Consequently, as humans are at the top of the food chain and depend on those things in which EDC accumulate and also cause ED, we will be the organisms affected most by biomagnification. It is then also this self-inflicted concern that is believed would one day drive the sixth mass extinction – the extinction of humanity. Whatever it might be first, resource limitation followed by the death of a species, diseased-state(s) followed by the death of a species or a combination thereof, the solution starts with us.

EDC modes of action (MOA)

The mechanism by which EDCs bring about ED is called the EDC‘s mode of action, or MOA. Currently the WHO leaves EDC MOA open-ended as results are still unclear (119). In an attempt to clarify some of the MOAs, two review articles by Tabb and Blumberg (120) and Swedenborg *et al.* (121) will be used to summarize current affairs.

Modulation of NRs through agonistic/antagonistic binding. By far the most widely studied EDC MOAs are the mechanisms through nuclear receptors (NRs) (120, 121) – specifically the estrogen receptors (ERs), androgen receptors (ARs) and thyroid receptors (TRs) (122). In these mechanisms, hormone-like EDCs bind the NR either as an agonist or antagonist, full or only partially, producing estrogenic, anti-estrogenic, androgenic, anti-androgenic, thyroidogenic or antithyroidogenic responses, consequently disturbing homeostatic regulation of endogenous hormones and ultimately causing a disease-state. An example of a NR modulator is diethylstilbestrol (DES) – a synthetic estrogen, renowned for its ability to mimic E_2 and activating the ERs. However, some EDCs, exhibiting NR modulation by mimicking the natural hormone, have been shown to also bring about dose-responses by ulterior means. An example is the plasticizer BPA. Although it mimics the dose-response of the natural hormone, E_2 in activating the ERs, closer examination of its effects on gene expression indicate that BPA does not stimulate the same set of genes as E_2 and therefore BPA is not a true estrogen, as it was designed to be (122).

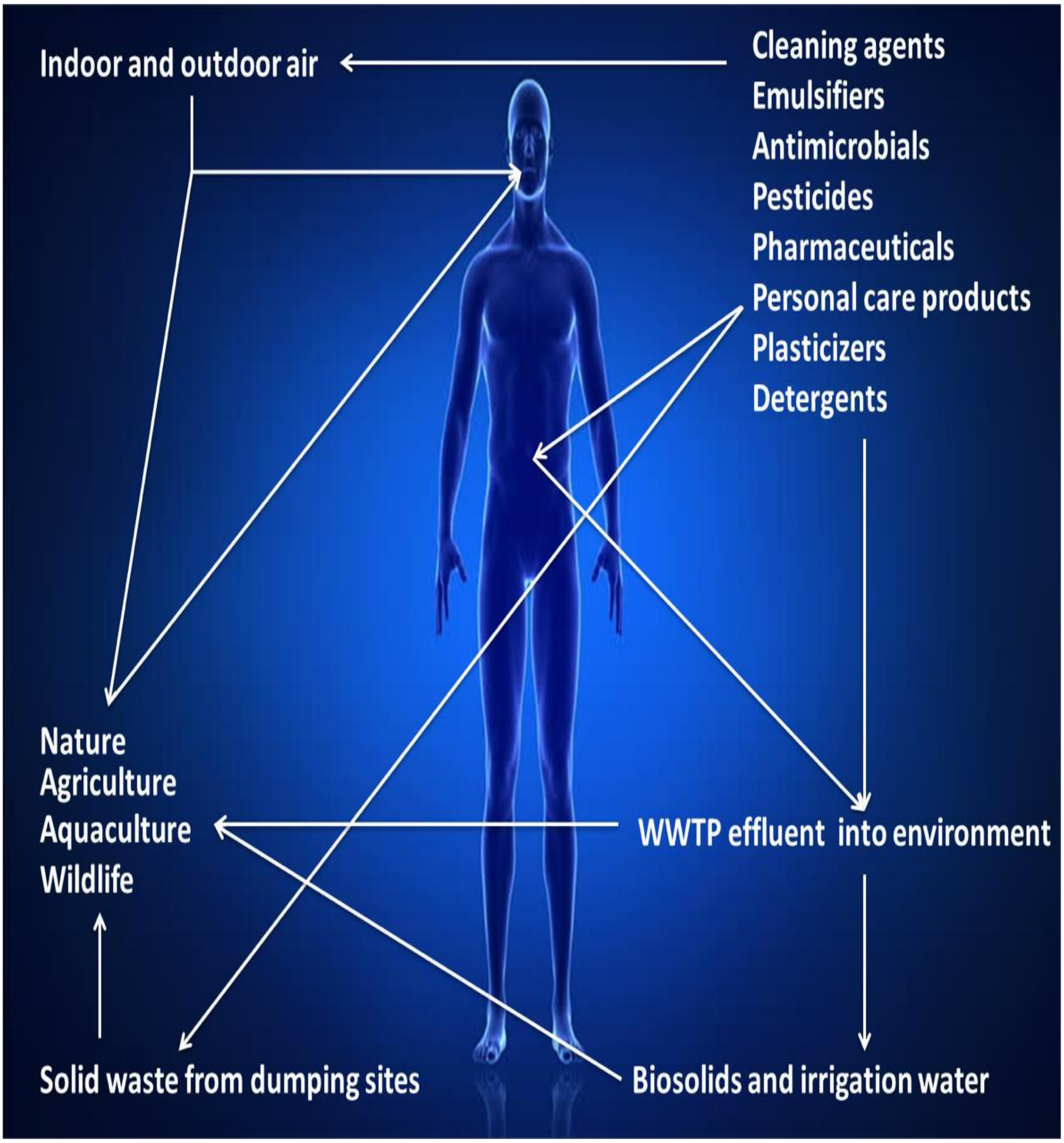

Figure 2.10 Routes of EDC exposure to humans. The various routes that EDCs can follow that ultimately affect animals and humans adversely.

Modulation of steroid hormone metabolism. Steroid hormone metabolism is a complex process involving multiple steroidogenic pathways, such as the classical, alternative, hydroxy-androstenedione and backdoor pathways (123–125). All steroids are derived from cholesterol, from which progestagens, mineralocorticoids, glucocorticoids, androgens and estrogens are synthesized by cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), aldoketoreductases (AKRs) and 5α-reductases (SRD5As), shown in Figure 2.11 (123).

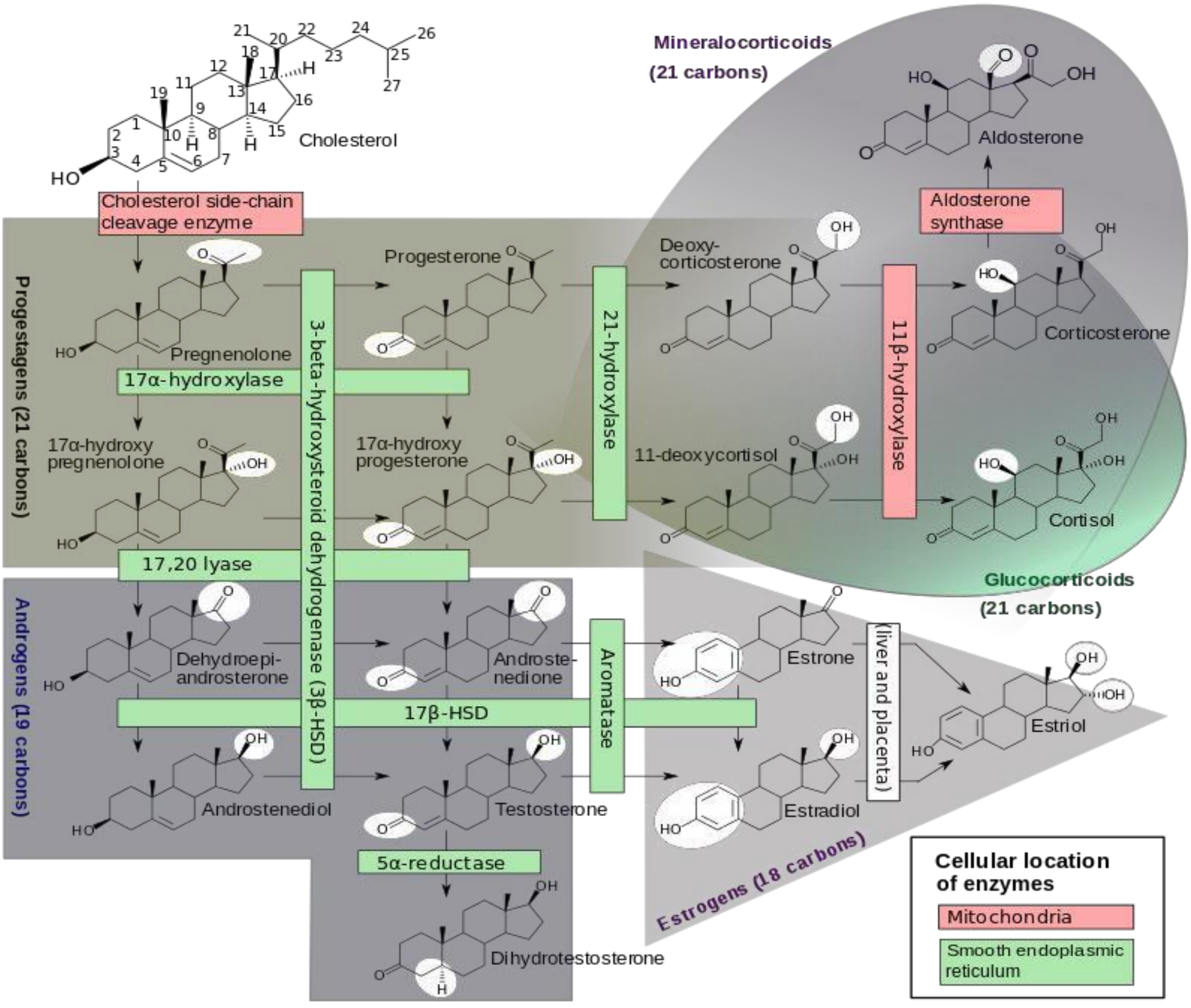

Figure 2.11 Steroidogenesis of the progestagens, mineralocorticoids, glucocorticoids, androgens and estrogens from cholestrol.

Availability of these steroid hormones depend on hormone biosynthesis, transport to the relevant target tissue, the amount of steroid hormone binding proteins and hormone catabolism. It is well known from literature that EDCs can alter all of the above mentioned processes (126–128). Two nuclear receptors, the human steroid and xenobiotic receptor

(SXR)/rodent pregnone X receptor (PXR) (129, 130) and constitutive androstone receptor (CAR) (131, 132), and a ligand-activated transcription factor (TF), aryl hydrocarbon receptor (AhR), have been implicated in modulating steroid hormone metabolism upon xenobiotic stimulation. SXR/PXR and CAR both regulate steroid hormone and xenobiotic metabolism (129–132). These receptors also mediate the induction of P450 enzymes such as CYP3A, CYP2B and CYP2C (133), as well as conjugation enzymes such as UGT1A1 (134) and the transporters P-glycoprotein, organic anion transporter peptide 2 and multidrug resistance-associated proteins (135). In contrast, AhR act on the genes of CYP1A2, CYP3A4, CYP1A1 and CYP1B1 responsible for the hydroxylation of E_2 (136). In addition, AhR also act on CYP19B (aromatase) responsible for converting testosterone (T) to E_2 (121).

A number of EDC have been shown to activate SXR/PXR, CAR and AhR. Schuets *et al.* (137) and Masuyama *et al.* (138) reported that certain phthalates, nonylphenol and organochlorides can activate mouse PXR and induce CYP3A. Similarly, Takeshita *et al.* (139) showed that BPA stimulate human SXR. Mikamo and colleagues (140) investigated the induction of CYPs by EDCs and found 54 EDC that is able to activate rat PXR and consequently induce the expression of CYP3A. Furthermore, classic EDCs such as Trans-nanochlor – a component of the currently band pesticide chlorodane, 1,1-dicloro-2,2-bis(ρ-chlorophenyl)ethylene – the metabolite of another band pesticide DDT and Methoxychlor – a substitute for DDT and structural analogue was reported to repress the basal activity of CAR in mice, increase both PXR and CAR transcriptional activity and activate SXR/PXR and CAR, respectively (141–143). It is hypothesised that the stimulation of CAR and SXR/PXR and the upregulation of their target genes increase the amount of EDC metabolites and consequently affect the bioavailability of endogenous hormones (120). Similarly, the activation of CYPs through the AhR by organic compounds such as dibenzofurans (PCDEs), polycyclic aromatic hydrocarbons (PAHs), dioxins, polychlorinated biphenyls (PCBs), benzofavone and benzo[a]pyrene (BaP), is believed to disturb endogenous hormone homeostasis (121, 144).

Modulation of NR co-activators. In the section on signal transduction through NR it was mentioned that co-activators are recruited after the NR binds to the HRE before transcription is initiated. These co-activators include members of the p160 family such as the steroid receptor coactivator-1 (SRC-1), glucocorticoid receptor interacting protein-1 (GRIP-1), amplified in breast cancer (AIB-1) and thyroid hormone receptor activator protein 220/peroxisomal proliferator-activated receptor gamma (THRAP220/PPAR-γ) (145–147).

In their 2002 article, Min and colleagues (148) showed that CAR can inhibit the transcriptional activity mediated through the ERs without ever binding to the estrogen response element (ERE). In their study overexpression of CAR caused a decrease in ER activity in a dose-dependent manner. Furthermore, treatment with a CAR agonist enhanced transcriptional repression mediated through CAR. When the authors then treated with androstenol – an antagonist of CAR, CAR-mediated transcriptional repression was relieved. From this it can be gathered that there exist competition between steroid receptors (SR) and xenobiotic receptors, and that the presence of xenobiotics mediating their effects through xenobiotic receptors could influence transcriptional regulation leading to ED. This corroborates finding from Reen and colleagues (149) where overexpression of both the AhR and ER co-activator-binding domain in mammalian cells caused AhR and ER impairments. This is not surprizing as competition for ARNT by AhR and ER has been shown to be partially responsible for the dioxin, 2,3,7,8-Tetrachlorodibenzo-p-dioxin‘s (TCDD‘s), anti-estrogenic properties (150, 151).

Furthermore, it has been suggested that disturbing the homeostasis of receptor and/or co-regulator mRNA and proteins would alter receptor activity (120). Inoshita *et al.* (152) showed that the EDC BPA increases THRAP220 and ERβ in mouse uterus. These results are similar to the findings of Lonard and co-workers (153), showing that selective ER modulators, hydroxy-tamoxifen (OH-TAM) and raloxifene, can increase steady-state NR coactivator levels, consequently boosting transcriptional activation of ERα. The results highlight the possibility that EDC can disturb receptor/co-regulator mRNA and protein homeostasis and thereby cause ED.

Proteasome-mediated degradation of NRs to interfere with hormone receptor activity. Proteasomes are protein complexes found throughout most domains of life. They play a major role in regulating intracellular proteins and peptides by proteolytic degradation, to e.g. remove old or damaged constituents, ensure controlled cell cycles by removing cyclins mediating cell cycle progression as well as removing peptide antigens displayed by the major histocompatibility complex (MHC) on antigen-presenting cells. These old or damaged proteins, cyclins or peptide proteins are recognized by the proteasome after being polyubiquitinated. Other cell constituents targeted by the ubiquitin-proteasome include several members of the NR family. This prevents endogenous hormones or other activating signals over stimulating cells. Therefore, inhibiting proteasomal degradation of these NRs could increase their concentration within a cell. This was shown by Sheflin *et al.* (154).

The receptors SXR/PXR are amongst the NRs regulated by proteasomal degradation. In a 2002 study Masuyama and colleagues (155) showed that the EDC phthalic acid can block the removal of PXR through proteasomal degradation compared to progesterone. This suggests that EDC can upregulate PXR protein levels by affecting PXR-mediated transcription. Recently Ohtake et al. (156) found that AhR and its heterodimerization partner, ARNT, are part of a ligand-dependent multiple-protein complex, 4B ubiquitin ligase, which plays a role in targeting proteins to the proteasome. This raises concern as AhR ligands could then interfere with hormonal signalling by proteasome-mediated degradation of SRs. In 1993 Wang and co-workers (157) found that an anti-estrogenic AhR ligand could decrease ERα levels through proteasomal degradation, supporting the claim that AhR ligands can interfere with hormonal signalling.

EDCs as hormone sensitizers. Chromosomes are packaged in chromatin that is folded around histones when not being transcribed. For histones to bind these chromatin structures histone deacytalases remove deacytal residues on histones to inhibit gene transcription. Work by Jansen *et al.* (158) showed that short chain fatty acids with no hormonal ability, valproic acid – an anticonvulsant and mood stabilizer, and methoxyacetic acid – a metabolite of methoxyethanol, increased ERα, ERβ, AR, PR and TRβ activity by inhibiting histone deacytalases. Their work suggests that a xenobiotic could sensitize NRs. This is particularly concerning as any hormone therapies, such as contraception or hormone replacement therapy (HRT), or the presence of other hormone-like xenobiotics, could amplify effects by this mechanism.

Interfering on DNA. It is thought that EDCs can bind to the AhR, which in turn binds inhibitory xenobiotic response elements (iXREs) situated close to the ER binding site, and thereby interfere with gene transcription (159). Hockings and colleagues (160) showed that the activated AhR could also bind XRE sites, normally occupied by unliganted AhR necessary for transcription, thereby abrogating gene induction by ER. However these findings need further investigation.

DNA methylation as a method for reprograming germ cells. Like histone deacytalation plays a role in gene transcription, so does the methylation of DNA. DNA methylation has been associated with key genetic processes such as X-chromosome inactivation, aging, carcinogenesis, genomic imprinting, and the repression of repetitive elements and is important in normal development. In primordial germ cells, DNA is demethylated and remethylated during gonadal sex determination. As correct remethylation is vital during this step any ED mediated through NRs by EDCs could reprogram the germ line.

Anway and co-workers (161) showed this MOA for methoxychlor and vinclozolin – a fungicide, when both these compounds altered, through DNA methylation, male germ cell spermatogenic capacity as well as the viability of sperm in rats. Rats further showed reduced fertility and lowered sperm development that was carried throughout the male germ line to the fourth generation. These results highlight the possible outcome of early exposure to EDCs.

Species specific effect. A particular interesting phenomenon was recently documented. Tabb and colleagues (162) found that highly chlorinated PCBs stimulated rodent PXR but antagonized the SXR of humans, consequently inhibiting gene expression involved in three phases of xenobiotic and hormone metabolism. The ability of these PCBs to have agonistic properties in one species and antagonist in another could point to a new MOA. The results, although interesting, could demerit EDC studies in toxicological model organisms.

EDCs as obesogens. Both Tabb *et al.* (120) and Swedenborg *et al.* (121) in their reviews acknowledge the role that EDCs play in acting as obesogens. It is known that certain NR play a crucial role in glucose uptake and fat metabolism and their dysregulation by EDC could contribute to obesity. In addition, AhR and its role in glucose homeostasis and lipogenesis have been shown by Thackaberry *et al.* (163) and Sato *et al.* (164),while the role of ARNT in diabetes-2 was established by Gunton et al. (165). It is hypothesized that loss ARNT expression coincides with impaired insulin release, leading to diabetes (165). Furthermore, the key regulator of adipose tissue, PPAR-γ, and its heterodimerization with RXR are known for their ability to regulate the genes involved in glucose and fatty acid metabolism as well as insulin signalling. Both PXR and RXR have been shown to be activated by organotins such as trybutyltin (TBT) and thereby induce adipocyte differentiation (166–168). Finally, EDCs such as PCB-77 has also been shown mediate adipocyte differentiation through the AhR, the surfactant, octylphenol, to increase the adipocyte specific hormone, resistin, which plays a role in insulin-resistance and BPA to increase pancreatic β-cell insulin as well as inhibiting adipocytes to release adiponectin (169–171).

Breast cancer

BC is the most common cancer in women worldwide and leading cause of cancer deaths in the US for women aged 20-59. In the US alone, new breast cancer incidences for the year 2016 were expected to reach 29% while the death toll was estimated to reach 14%. Globally, 15% of cancer-related deaths in women are attributed to breast cancer (172, 173). However, according to US cancer statistics only 5-10% of BCs can be linked to gene mutations, such as *BRCA1* and *BRCA2*, (174). Moreover, 85% of women with breast cancer have no family history (174). These statistics thus raises the question: If not genetic and there exists no family history, what then causes BC?

Anatomy and physiology of the human breast. Anatomically, the breast area can be divided into four quadrants (Figure 2.12), namely, the upper inner and outer quadrants (UIQ and UOQ), and lower inner and outer quadrants (LIQ and LOQ). Studies have shown that 66% of breast tumours occur in the outer quadrant of the breast (175–177). Female breast is mostly made up of adipose tissue that covers the breast lobes, containing lobules, and milk ducts. The ductal system is covered by epithelial cells, thought to be the site where most breast cancers originate from (178). Each lobule contains milk secreting alveoli consisting of a lumen, surrounded by alveolar epithelial cells and myo-epithelial cells. Within the adipose tissue there are also ligaments, fibrous connective tissue, nerves, lymph vessels, lymph nodes and blood vessels. Together these systems maintain a healthy breast necessary for possible parturition.

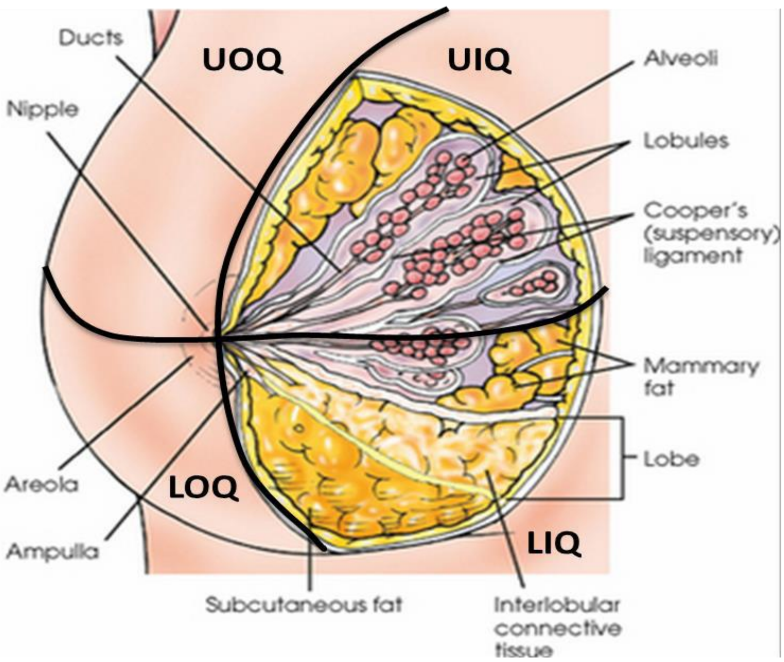

Figure 2.12 Frontal view of the human breast divided into four quadrants. UOQ, Upper outer quadrant; UIQ, Upper inner quadrant; LIQ, Lower inner quadrant; and LOQ, Lower outer quadrant.

Throughout developmental the breast undergoes dramatic changes in shape, size and function. However, it is only during puberty that lobule formation starts and at the end of the first full term of pregnancy that the breast is fully matured. During all stages of development estrogens – estrone (E_1), estradiol (E_2), estriol (E_3) and estetrol, and progesterone play a central role in the proliferation and differentiation of normal breast epithelium. Of these estrogens estradiol is the most common estrogen and most abundant estrogen form in nonpregnant women. In contrast, estriol is most commonly found in pregnant women. Estrone, on the other hand, is primarily detected in post-menopausal woman (179). It is also believed that during the stages of gestation, puberty and pregnancy women are particularly susceptibility to breast cancer where exposure to xenobiotics such as EDCs can cause irreversible and cross-generational effects (180). These hormones thus represent the different stages of a female‘s life and are key regulators during development. Deregulation of the estrogens and progesterone are therefore thought to be responsible for BC development (178, 180, 181).

Breast cancer types. Histologically, BC can be grouped into four molecular subtypes, namely, luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) type and triple negative, corresponding to their ER, HER2 and PR status. Here ER-, PR- or HER2-positive refers to a BC cell expressing the ER, PR or HER2 receptor and therefore is responsive to estrogens, progestens or the human epidermal growth factor. One other factor, Ki67 – an antigen serving as a cellular marker for proliferation, is also used to distinguish the different types. Luminal A is by far the most common BC (30-70%) and has a HER-, ER+ and PR- or ER- and PR+ receptor status. These BC cells are normally responsive to hormone therapy. In contrast, luminal B only presents itself 10-20% of the time, with a HER2+ or HER2- receptor status with high levels of Ki67. In addition, luminal B also have an ER+ and/or PR+ receptor status. The HER2 type presents itself 5-15% of times and have ER-, PR-, and HER2+ receptor status. These BC are treated with drugs such as Herceptin. Lastly, triple negative/basal-like BC presents itself 15-20% of times and is unresponsive to hormone therapy as all receptor statuses are negative. Treatment normally involves surgery, radiation and chemotherapy (182–186).

Factors influencing the initiation, progression and reoccurrence of breast cancer. The Centres for Disease Control (CDC) attributes breast cancer to several factors (Table 2.2). Some of these factors, such as HRT, contraception, early menstrual period and the use of DES is the direct result of ED, and therefore these causes should be attributed to ED. BC are mainly attributed however to loss of function of *BRCA1*/*BRCA2* genes, diet, smoking, alcohol, radiation and a lifetime exposure to estrogens (Lipworth (187), Key *et al.* (188) and Yoshida and Miki (189)). In 1995 Lipworth's epidemiological study showed that 90% of BC cases are environmental in origin (187). This started an era of research dedicated to linking xenobiotics to health effects.

Table 2.2 Factors attributed to breast cancer. Adapted from the CDCs website (190)

Age	Genetic mutations	Early menarche
Late/no pregnancy	Not being active	HRT
Contraception	Personal history of BC	Family history of BC
Radiation therapy	History with DES	Alcohol

Most reports until 2003 investigated the link between BC and environmental estrogens that enter the body through food, water and air (191–194). However valuable these studies have been, some of the results cannot be extended to the high amount of xeno-estrogens present in the breast or the ability of these xeno-estrogens to migrate to the breast through food, water or air, where they can have their adverse effects. In 2001 Darbre started investigating underarm cosmetics as the cause of breast cancer (195), showing that long-term exposure, to a variety of chemicals, at regular application intervals, of underarm cosmetics to the underarm breast area significantly increase BC susceptibility (178, 196, 197). This is in agreement with the earlier statement that 66% of breast tumours occur in the outer quadrant of the breast. Darbre and colleagues have since extended the list to personal care products (PCPs) (198). Some of these PCPs and their relevance to BC will be discussed in the section on common EDCs and their involvement in breast cancer.

The hallmarks of cancer: setting the stage for the role of EDCs in breast cancer. Cancer is an extremely complex disease. To better understand its complexity it is often reduced to a number of ‗hallmarks' first suggested by Hanahan and Weinberg (46, 199). In the two publications on ‗The hallmarks of cancer' the authors reduce the complexity of cancer development down to four basic underlying principles in addition to two cancer enabling hallmarks. One, cancer cells cause sustained proliferation. Two, cancer cells can evade growth suppression and induce angiogenesis. Three, cancer cells can resist cell death. Four, cancer cells have the ability to activate invasion and metastasis. In addition, cancer cells have increased genomic instability and are able to reprogram energy metabolism.

Since being first described in 2000 these hallmarks have been regarded as the gold standard for deciding the potential threat of chemicals, i.e. whether or not a chemical could cause cancer. In 2013, Sonnenschein and Soto (200) criticized the hallmarks for inconsistencies in data, ignored data, viewing cancer as a cell-based genetic disease, and the fact that their explanation of cancer phenotypes is based on the somatic mutation theory (SMT), namely, the theory that cancer is the consequence of mutations in body cells rather than germ cells, and its cell-centred variants. Instead the authors argue that cancer should be viewed as a tissue-based disease where the default states of all cells are proliferation and motility. Furthermore, carcinogenesis should be viewed as a miscommunication of reciprocal interactions between cells and their extracellular matrix.

Nevertheless, the hallmarks of cancer proposed by Hanahan and Weinberg (46, 199) serve as good characteristics of cancer when describing the ability of anthropogenic chemicals to modulate endogenous hormones necessary for normal cellular development. Therefore, the next section will deal with common EDCs and their involvement in breast cancer as if they can contribute to these hallmarks.

Common EDCs and their involvement in breast cancer

Pesticides. Pesticides are diverse group of chemicals which include herbicides, avicides, nematicides, repellents, insecticides, molluscicides, rodenticides, antimicrobials, disinfectants, fungicides, miticides, defoliants and desiccants (201). Generally defined as any substance or mixture applied to soil, water, plants, crops, animals, structures, clothing and furnishing, these substances attract or repel, kill, interrupt or regulate their targets (201). However, many of these substances have been shown to leach into ground or river water where they affect non-targeted organisms in the environment (202). Evidently, these compounds then also pose a risk to third parties, such as humans, that live in and depend on the affected environment for food. A case study by London and colleagues (203) linked organophosphate exposure under farm workers to an increased risk of suicide, emphasizing the effect on non-targeted organisms.

Probably one of the best described pesticides having ED properties is dichlorodiphenyltrichloroethane (DDT). First synthesized in the 1870s, this organochloride was used as an insecticide to control malaria and typhus during World War II. However, during the 1950s animal studies suggested that DDT influence reproductive success (204, 205). These results were also discussed in the very controversial book, Silent Springs, by Rachel Carson (206). By the 1970s DDT was shown to accumulate in predatory animals higher up in the food chain (207). Later it was shown that DDT and its metabolites have estrogenic potential (208) and act as an androgen antagonist (209). In 1992 Wolff and Toniolo linked DDT to breast cancer (210). However, work by Lopez-Carillo et al. (211) did not support the findings of Wolff and Toniolo. In 2015, a case-control study by Cohn and colleagues (212) again showed that DDT exposure increased the risk of developing BC.

Since the use of DDT a number of studies have linked other pesticides to diseased-states. These include leukaemia (213, 214), lymphopoietic cancers (215), prostate cancer (216, 217), non-Hodgkin's lymphoma (218), brain cancer (219), liver cancer (220), pancreatic cancer (221), multiple myeloma(222), diabetes (223, 224) and developmental effects (225). As pesticides are widely used today and so many of their active metabolites linked to some kind of ED, it is recommended that ED studies be performed before being released on the market.

Industrial EDCs. The range of industrial EDCs is large and diverse. Therefore, this part will only focus on the members of most concern that includes plasticizers, plastic intermediates, alkylphenol ethoxylate precursors and industrial coolants.

Plasticizers are the collective term used to describe additives added to a variety of materials for enhanced plasticity or fluidity. They can be added to plastics, concrete, gypsum wallboard and energetic materials such as rocket propellants and smokeless powders to enhance the elasticity and structure of commercial products. The most common plasticizers include trimellitates – used in the automotive industry or where resistance to high temperatures are needed, adipates and sebacates as well as maleates – used where resistance to low temperatures and UV are needed, biodegradable citrate plasticizers and phthalates. Many of these, particularly the latter group, phthalates, have been shown to be EDCs.

Phthalates are lipophilic compounds used primarily in the production of polyvinyl chloride (PVC), nitrocellulose and polyvinyl acetate (PVA), medical products, toys, plastic coatings, cosmetic formulations and food wraps etc. (226). These compounds have been shown to be ubiquitously present in the environment and able to be absorbed through the skin, to cross the placenta (227–229) as well as accumulate in breast milk (230–232). Moreover, these compounds have shown numerously to be potent endocrine disrupters exhibiting anti-androgenic activity in animal models (233), reduce reproductive organ weight and sperm count in male rodents, prolong estrous cycles and lowering circulating E_2 in female rodents (234–237) as well as cause the late onset of menarche (238). Their role in breast cancer is also clear as these compounds mimic the natural hormones and bind ERs (19, 239, 240) and induce the expression of c-Myc – a regulatory gene important in cell cycle progression, cellular transformation and apoptosis, in ER- BC cell lines (241). In addition these compounds have also been shown to increase MCF-7 BC cell proliferation (226, 240) and mediate the activations of PPAR-α (242). In addition phthalates can also inhibit Tamoxifen-induced apoptosis in MCF-7 BC cells (226), reducing the effectiveness of endocrine therapy. It is therefore no shock to find that phthalate use in consumer products is restricted in both Europe and the United States.

Another well-known is plasticizer with ED properties is the compound BPA. It is primarily used in the production of polycarbonate plastics and resins used for the packaging of beverage bottles and canned foods. A number of studies found that BPA can leach from the linings of food and soda cans, polycarbonate bottles and dental sealants, respectively (243–245). This is particularly concerning as BPA has been shown to increase the susceptibility of rats to develop prostate cancer and at low doses also reduce their sperm count. Additionally, BPA could increase prostate gland weight in male rats, while in also disturbing the development and tissue organization of the mammary gland. Moreover, BPA was found to cause deleterious effects in the vagina, accelerated growth and puberty as well as disrupt sexual differentiation in female rats (246–251). Hunt and colleagues (252) showed that BPA can also disrupt meiosis in mouse. Furthermore, BPA exposure has also been associated with obesity (77, 253, 254) and diabetes (255). Like phthalates BPA can also mimic endogenous hormones and bind the ERs (256). Lastly, BPA have been shown to target the mammary gland (256) where it can alter architecture of a mature breast *in utero* (68).

Like BPA, nonylphenols are alkylphenols, with the most widely being used being 4-nonylphenol (257, 258). 4-Nonylphenol is primarily used in the production of alkylphenol ethoxylates (APEs) that are used as wetting, dispersing, emulsifying and stabilizing agents in agricultural, domestic, industrial and consumer products (259, 260). Also able to leach from their final products, 4-nonylphenol is a persistent organic pollutants often detected in the environment (261–263). As with BPA, these compounds have also been linked to both obesity and diabetes (77, 253, 254). Furthermore, 4-nonylphenol has been found to cause ED by mimicking the natural hormone E_2. In doing so, 4-nonylphenol has been shown to interfere with the estrous cycle of rats as well as altering pubertal onset (264, 265). In addition, it can also cause proliferation of both estrogen-dependent and estrogen-independent breast cancer cells (266), indicating that this compound can modulate several physiological pathways. In pregnant women, even al low doses, 4-nonylphenol has been found to increase apoptosis and cell death in the cells of the placental. Moreover, treatment with 4-nonylphenol resulted in an increase in the secretion of interleukin (IL)-4 and IL-10, reduced tumour necrosis factor alpha (TNFα) and increased interferon gamma (INFγ), *in vitro* of first trimester placental cells. This marked unbalanced cytokine profile is linked to implantation failure and pregnancy loss (267).

Another group of EDCs commonly associated with cancer is polychlorinated biphenyls (PCBs). PCB‘s are lipid soluble persistent environmental pollutants used as dielectric fluids in capacitors and transformers, plasticizers, adhesives, organic diluents and flame retardants (268, 269). The group consist of 209 PCB congeners of which most have been linked to ED (268). These organochlorides are widely studied for their ability to act as a carcinogen, estrogen mimic, immune disruptor, modulator of cellular communication and inducer of human cytochrome P-450 (CYP) genes (270–273). In rats PCBs have been shown to cause liver cancer and in humans suggested to lead to the progression of BC (192, 274, 275). A study done by Liu *et al.* (276) showed that PCBs can increase the motility of both non-metastatic MCF-7 BC cells as wells as metastatic MDB-MB-231 BC cells. As metastasis in a fundamental characteristic of cancer cells, these properties link them to BC progression. However, PCBs doesn‘t only act as carcinogens, Brouwer and colleagues (277) showed that PCBs could also influence thyroid homeostasis by reducing their concentration of thyroid hormones in experimental animals.

In conclusion, industrial by-products are shown to exert several mechanisms of ED by altering several physiological pathways within vertebrate endocrine systems.

Pharmaceuticals. Many people consider pharmaceutical usage as safe when prescribed by a health practitioner. However, it is quite evident from most drug leaflets that medication can have severe side effects. In most cases, the longer a drug is on the market, the more adverse side effects are noted. However, as these side effects only get noticed over time, by the time a person has used the medicine multiple times, the adverse side effects have already run its course. A good example of a well-intended medicine, now an EDC, gone awry is the drug diethylstilbestrol (DES). Prescribed to women over the period 1938-1971, this synthetic non-steroidal estrogen was meant to prevent miscarriages and contribute to a healthy pregnancy as well as to treat gonorrhoeal and atrophic vaginitis, postpartum lactation and menopausal symptoms (278). However, in woman taking DES prescriptions vaginal adenocarcinomas and an increased risk of BC was found (279–281). In addition DES daughters displayed an increased risk of cervical intraepithelial neoplasms, leukoplakia, mosaicism, clear cell adenocarcinomas, infertility and complication during pregnancy (279). Moreover, epididymal cysts, variococele and hypoplastic and undescended testes were observed in DES sons (282). From this it is clear what the possible outcome could be if a drug is not properly researched.

An emerging environmental pollutant, detected regularly in aquatic systems around the world (283–285), and EDC is the anticonvulsant carbamazepine (CBZ). Primarily used to treat neuropathic pain, schizophrenia, bipolar disorder and epilepsy, this drug is the most widely used first-line anti-epileptic drug (AED). Studies done on patients treated with CBZ found that both men and women are prone to sexual dysfunction and reproductive endocrine disorders when taking this drug. In men, reduced potency of sperm as well as sperm abnormalities was noted (286–288), while women displayed menstrual disorders, polycystic ovaries and infertility (288–293). It is thought that CBZ causes AED-induced androgen deficiency by either inducing the synthesis of sex hormone binding globulin (SHBG) – the glycoprotein that binds androgens and estrogens, and thereby reduce the amount of loosely bound or unbound hormones, or, CBZ increases serum E_2 levels, which in turn suppress the synthesis of testosterone through a negative feedback loop (287, 288, 294–296). This is supported by findings from a number of studies that found CBZ-therapy was associated with lowered levels of dehydroepiandrosterone sulfate (DHEAS) (297–299). However, Perucca and coworkers (300) argued that the decrease is the result of induced CYPs. CBZ was also shown to alter the behaviour and growth of certain algae, fish and cladocerans (301–305), developmental abnormalities (306), modifications to fecundity (303, 305, 307) and histological changes in gonads and decrease levels of testosterone (308). Conversely, similar results obtained for CBZ was found for three other anti-epileptic drugs – Levetiracetam, Lamotrigine and Valproate.

Personal care products. The term personal care products (PCPs) refer to any/all products used by consumers to enhance their personal well-being, including but not limited to, sun screens, toothpaste, antiperspirants, deodorants, roll-ons, soaps, detergents, body wash, lotions and cosmetics. Like pharmaceuticals, consumers often consider the use of these products as safe. Recently it has come to light that some active ingredients in PCPs have endocrine disrupting properties, many of which are shown to cause proliferation of breast cancer cells *in vitro*. Particularly, research has shown the potential of three PCP preservatives to modulate endocrine system pathways, namely triclosan (TCS), triclocarban (TCC) and parabens. Of note, in September 2016 both TCS and TCC was classified as NOT generally recognized as safe and effective (GRAS/GRAE) by the FDA and therefore their use in consumer antiseptic washes banned in the USA (309).

TCS is a chlorinated aromatic additive with bactericidal properties, present at 0.1-0.3% (w/w) of PCPs (310, 311). Its frequent use in PCPs is evident from WWTP concentrations reaching the parts per billion (ppb) to parts per million (ppm) range (312, 313). A recent study by Calafat and colleagues (314) found that TCS is present in 75% of urine samples taken from US citizens. Moreover, TCS has further been shown to accumulate in breast milk (315), therefore creating the potential to bio-accumulate in lactating offspring. However, TCS has also been detected in women with no prior exposure to PCPs containing TCS (310, 316), suggesting there might be background exposure such as contaminated water. Due to the structurally similar conformation of TCS to thyroid hormones (317), its presence in vertebrates could have detrimental effects. TCS has been shown to accelerate metamorphosis in North American bullfrogs (*Rana catesbaenia*), as well as to decrease TRβ mRNA expression (318), reduce plasma testosterone and vitelloginin levels in the African clawed frog (*Xenopus laevis*) (319), inhibit the activity of rat and human microsome iodothyronine (T_2) sulfotransferase (320) and decrease circulating T_4 levels in rats (317). With regards to its ability to contribute to BC, TCS was found possess both intrinsic estrogenic and androgenic properties (321), able to bind the ER and thereby induce cell proliferation (321), and increase the expression of cyclin D1 and decrease the expression of p21 in MCF-7 BC cells (322). However contrasting results have also been noted as TCS have been found to inhibit fatty acid synthase (FAS) – overexpressed in certain cancers (323–326), as well as inhibits tumour development in rats fed with a diet supplement of a 1000 ppm TCS (327). From these studies it is evident that TCS might seriously contribute to ED and pose risk to BC, but more data is needed to support its link to the development of BC as contrasting findings suggest it might act as an anticancer drug or at least a cancer sensitizer.

TCC is a TCS analogue with an identical biocidal activity as TCS. In contrast to TCS, TCC is used at higher concentration – typically 2% (w/w). However like TCS, TCC is also a commonly used in PCPs (328). Work by Ahn *et al.* (329), Chen *et al.* (330) and Sood *et al.* (331) showed that TCC on its own lacks the ability to induce ER-responsive gene expression but in combination with E_2 stimulates the expression of ER-responsive genes. Sood and colleagues also found that a single TCC exposure could activate the Erk-Nox pathway, increase cell proliferation, cause the elevation of reactive oxygen species (ROX) and DNA damage in MCF10A cells. In contrast, repeated exposure induced transient cancer-like characteristics in MCF10A cells such as reduced dependence on growth factors (RDGF) and anchorage-independent growth (AIG). Additional repeatedly exposed to TCC showed constitutive endpoints, including RDGF, AIG, cell proliferation, elevated ROS levels and Mek-Erk-Nox pathway activation. TCC is therefore considered to be an EDC with carcinogenic properties. Finally, TCC has been shown to be also able to disrupt thyroid hormone-responsive gene transcript levels in GH3 cells (332).

Another group commonly used as preservatives are the parabens. These chemicals are esters of parahydroxybenzoate and are added to PCPs to a final concentration of no more than 0.8% (w/w). Of all the parabens the most commonly used in PCPs are methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP) and butylparaben (BuP), of which MeP and PrP are the most likely to be found in PCPs, either alone or in combination with one another. Like TCS and TCC their frequent use is evident from their widespread detection in WWTPs and other aquatic systems. However these compounds have also been detected in urine (333–336), blood (337), semen (335, 338), breast milk (339, 340), breast tissue (341) and breast tumours (342). Their widespread distribution throughout the human body has raised concern even before Darbre and colleagues started investigating their link to ED and BC in 2002 (343–349). Since then parabens have attracted much attention for being present in tissue at concentrations equivalent to the amount needed for ER-responsive proliferation (350, 351) and their ability to stimulate not only MCF-7 BC cells but also MCF10A cells at low concentrations (351). In addition, parabens have not only been shown to induce both *ESR1* and *ESR2* gene expression (349, 352, 353) but also increase CYP19A1 gene and protein expression in MCF-7 BC cells (351).

Individually, PrP and BuP increase PR mRNA while MeP and PrP increase PR protein expression in MCF-7 BC cells and MCF10A cells (354). PrP have been found to increase plasma vitellogenin levels in Japanese medaka fish (355), while BuP and PrP at low doses could alter sperm production in rats and mice as well as cause DNA fragmentation, chromosome alterations and increase sister-chromatid exchanges in treated Chinese hamster ovary (CHO-K1) cells (356). Lastly, MeP was found to not only upregulate the mTOR pathway in human breast epithelial but also prevented OH-TAM from suppressing breast cancer cell proliferation (357). Together, these properties show that parabens can act as EDC, carcinogens, BC progressors and act as BC treatment effectors, enough evidence to suggest their use in consumer PCPs are NOT GRAS/GRAE and therefore should also be banned, like the case is with TCS and TCC.

2.5.6 Summary

The sections above emphasized the importance of maintaining homeostasis and that disruption of the endocrine system could have adverse effects. Section 2.5.3 and 2.5.4 discussed the role that the HPA, HPG and HPT axes play in maintaining homeostasis and how the hormones controlled by these axes play a central role in signal transduction to bring about effects such as the proliferation of cells. The last section (section 2.5.5) stressed how certain compounds can partake in uncontrolled signal transduction or interfere with signal transduction and that this disruption can be the causative effect behind diseases such as breast cancer. Finally, the sections also discussed the various routes that humans can be exposed to EDCs, with the environment that we find ourselves in playing a major role.

CHAPTER 3

DEVELOPING ANALYTICAL TECHNIQUES FOR THE PRE-CONCENTRATION, IDENTIFICATION AND QUANTIFICATION OF 9 COMMONLY OCCURRING POLLUTANTS WITH ED CAPABILITIES FROM ENVIRONMENTAL MATRICES

3.1 Introduction and chapter objectives

In chapter 2 section 2.3 and 2.4 the methods for monitoring pollution were discussed. It was concluded that the biological assays can indicate the presence of pollution but that these do not reveal the specific pollutant(s) present. However, the analytical chemistry methods discussed can both indicate pollution and identify the type of pollutant. Although these methods UPLC-, UPC^2- and GC-MS/MS, have the advantage of specificity, their initial setup on the machines used is expensive, time-consuming and require a great deal of knowledge and experience. However, once machine setup has been validated their use become significantly less expensive, have a high sample throughput and does not require in-depth knowledge. This chapter reports on the aim to develop methods on the above mentioned systems that can be used to evaluate the JTED Eco-machine. The chapter aims and objectives are as follow:

a) Develop and optimize methods on the identified instruments for common EDCs
b) Determine the analytical capabilities of each instrument and thereby the best suitable analytical instrument
c) Develop an EDC method capable of pre-concentrating the majority of identified EDCs from water or synthetic waste water (SWW) matrix
d) Validate the pre-concentration, identification and quantification method

3.2 Results

3.2.1 Eleven commonly occurring EDCs were selected for monitoring the efficiency of the Ecomachine and the state of water pollution by GC-, UPLC- and UPC^2-MS/MS analysis.

To monitor the efficiency of the Ecomachine and the state of EDC pollution in the Berg River, 11 commonly occurring EDCs and one human indicator were selected. Selection was based on already available EDC standards in the lab, most studied EDCs, the severe consequences (see section 2.5.5) if found in nature and possible contribution to breast cancer or the recurrence thereof. These EDCs include the three human estrogens – E_1, E_2 and E_3, the synthetic E_2 analogue used as contraceptive – EE_2, the four common PCP preservatives relevant to chapters 4 and 5 – MeP, PrP, TCS and TCC, the alkyl phenols BPA and 4NP, and the anticonvulsant CBZ. Additionally, paracetamol (PCM) was used as human indicator. To account for variation in sample preparation during the determination of each instruments analytical capability, development of the EDC pre-concentration method from water and SWW matrices and final validation, three radio-labelled internal standards (IS) – 4NP-d_4, E_2-d_5 and CBZ-d_{10} were chosen. Finally, three analytical instruments readily available for analysis were selected. These include a Thermo Fischer GC coupled to an EI-MS/MS and a Waters UPLC and UPC^2 coupled to the same ESI-MS/MS. All three instruments are considered state-of-the-art. The mass spectrometer coupled to both the UPLC and UPC^2 is newly developed by Waters with tandem quadrupole S-wave (TQ-S) technology that is able to remove non-ionized compounds before proceeding to the detector, effectively decreasing background noise normally associated with non-ionized compounds. Seeing that the same mass spectrometer is used for UPLC and UPC^2, setup was carried out once and the same precursor-to-product (transitions) used for both UPLC-MS/MS and UPC^2-MS/MS. To improve the volatility and ionization of certain compounds during analysis, compounds were derivatized with DSCl (UPLC/UPC^2) or BSTF (GC). The optimized MRM setup for UPLC/UPC2-MS/MS can be found in Table 3.2, while the optimized SRM setup with retention times for GC-MS/MS is displayed in Table 3.3. Retention times for UPLC/UPC2 are shown in Table 3.1.

Table 3.1 Retention times (min) of the different compounds when separated with UPLC or UPC^2 on the respective columns described in materials and methods.

Compound	UPLC-MS/MS	UPC^2-MS/MS	
	BEH	BEH 2-EP	Torus 2-PIC
CBZ	1.16	2.46	4.27
CBZ-d_{10}	1.15	2.45	4.26
PCM	1.80	6.12	7.83
E_3	2.90	8.36	8.85
TCC	2.85	6.12	9.28
MeP	2.83	1.25	1.40
PrP	5.14	1.19	1.37
E_2	5.37	5.82	7.20
E_2-d_5	5.33	5.81	7.17
EE_2	5.60	5.73	7.44
E_1	5.70	3.11	3.80
TCS	6.93	1.70	2.27
BPA	7.54	5.36	5.99
4NP	8.61	1.31	1.55
4NP-d_4	8.59	1.30	1.54

Table 3.2 MRM transitions of each compound used in both UPLC- and UPC^2-MS/MS analysis. Product ions printed in bold were used as quantifiers while their respective partners were used as qualifiers. Internal standards: 4NP-d_4, E_2-d_5 and CBZ-d_{10}. DND, did not derivatize; CV, cone voltage; CE, collision energy.

Compound	ESI mode	Precursor	CV (V)	Product ion 1	Product ion 1 CE (eV)	Product ion 2	Product ion 2 CE (eV)
CBZ	+	237 (DND)	25	179	25	**194**	15
CBZ-d_{10}	+	247 (DND)	30	202	15	**204**	15
PCM	+	385	35	156	35	**171**	20
E_3	+	522	35	156	55	**171**	35
TCC	-	313 (DND)	20	126	20	**160**	25
MeP	+	386	35	156	40	**171**	20
PrP	+	414	35	**156**	40	171	35
E_2	+	506	35	156	40	**171**	35
E_2-d_5	+	511	35	156	50	**171**	30
EE_2	+	530	40	156	40	**171**	35
E_1	+	504	20	156	45	**171**	35
TCS	+	522	20	156	55	**171**	35
BPA	+	695	50	170	35	**171**	35
4NP	+	454	20	156	40	**171**	20
4NP-d_4	+	458	30	156	50	**171**	30

Table 3.3 SRM transitions and retention times of each compound used in GC-MS/MS analysis. Quantifier transitions are printed in bold. N/A. not applicable; DND, does not derivatize. R_t, retention time; m/z, mass to charge; CE, collision energy.

Compound	Underivatized mass	Derivatized mass	Precursor 1 (m/z)	Product ion 1 (m/z)	Product ion 1 CE (eV)	Precursor 2 (m/z)	Product ion 2 (m/z)	Product ion 2 CE (eV)	R_t (min)
CBZ	236.1	DND	193	165	10	**193**	**193**	12	20.88
CBZ-d_{10}	246.1	DND	203	175	10	**203**	**203**	12	20.80
PCM	151.1	295	**280.3**	**206.1**	10	295	206.1	10	11.56
E_3	288.4	360	**386.2**	**296.2**	10	504.2	414.2	8	22.63
TCC	314.0	DND	**296**	**226**	10	298	228	10	14.25
MeP	152.0	224	209.1	177.1	8	**224.1**	**209.1**	8	10.51
PrP	180.1	252	**210.1**	**195.1**	8	252.1	195.1	14	12.51
E_2	272.4	416	**416.2**	**282.2**	12	416.2	326.1	8	21.60
E_2-d_5	277.4	421	**421.3**	**287.4**	15	421.3	331.3	8	21.56
EE_2	296.2	440	**425.2**	**425.3**	8	440.3	425.3	10	22.60
E_1	270.4	342	342.2	218	12	**342.2**	**257.2**	12	22.92
TCS	288.0	360	**345**	**200**	12	347	202	8	17.83
BPA	228.1	372	**357.2**	**191.1**	16	372.2	257.2	12	17.77
4NP	220.2	292	292	292	8	**292.2**	**179.1**	10	14.44
4NP-d_4	224.2	296	296	296	8	**296**	**183**	8	14.35

3.2.2 Instrument method optimization

After initial setup on each instrument, a number of variables were investigated that can affect the overall performance of each instrument to detect and quantify compounds. For UPLC- and UPC^2-MS/MS, derivatizing solvent composition, mobile phase flow rate, column temperature (excl. UPC^2-MS/MS), and desolvation gas temperature and flow rate were considered. In contrast, derivatizing solvent composition, EI emission current and Q1 resolution, and split/splitless mode were considered for GC-MS/MS. To assess the influence of each variable on the overall performance of the instrument the parameters peak area, peak shape (b/a) and signal-to-noise (S/N) were monitored. Variable influences on the different instruments are shown in **Error! Reference source not found.** - **Error! ference source not found.**. The optimized method for UPLC-MS/MS, UPC2-MS/MS and GC-MS/MS are shown in Table 3.4, Table 3.5 and Table 3.6, respectively. Unfortunately during the optimization process it was found that the compounds 4NP and CBZ gave unreliable results and were excluded from the rest of the study.

Table 3.4 UPLC-MS/MS method optimization. A, Acetone; B, 0.1 M $NaHCO_3$ buffer (pH 10.5); C, Acetonitrile.

Variable Tested	Before optimization	After optimization
Derivatizing solvent composition	50% A, 50% B	75%C, 25%B
Mobile phase flow rate	0.35 mL/min	0.4 mL/min
Column Temperature	50 °C	60 °C
Desolvation gas Temperature	500 °C	550 °C
Desolvation gas flow rate	900 L/Hr	1000 L/Hr

Table 3.5 UPC^2-MS/MS method optimization. A, Acetone; B, 0.1 M $NaHCO_3$ buffer (pH 10.5); C, Acetonitrile.

Variable Tested	Before optimization	After optimization
Derivatizing solvent composition	50% A, 50% B	75%C, 25%B
Mobile phase flow rate	1.8 mL/min	1.7 mL/min
Desolvation gas Temperature	500 °C	500 °C
Desolvation gas flow rate	800 L/Hr	800 L/Hr

Table 3.6 GC-MS/MS method optimization. SPLESS. splitless; PSPLESS, pulsed splitless; P, pyridine

Variable Tested	Before optimization	After optimization
Derivatizing solvent composition	67% P, 33% BSTFA	67% P, 33% BSTFA
EI emission current	50 µA	50 µA
Q1 Resolution	Wide (1.5 at 50%)	Wide (1.5 at 50%)
Injection (PSPLESS/SPLESS)	PSPLESS	PSPLESS

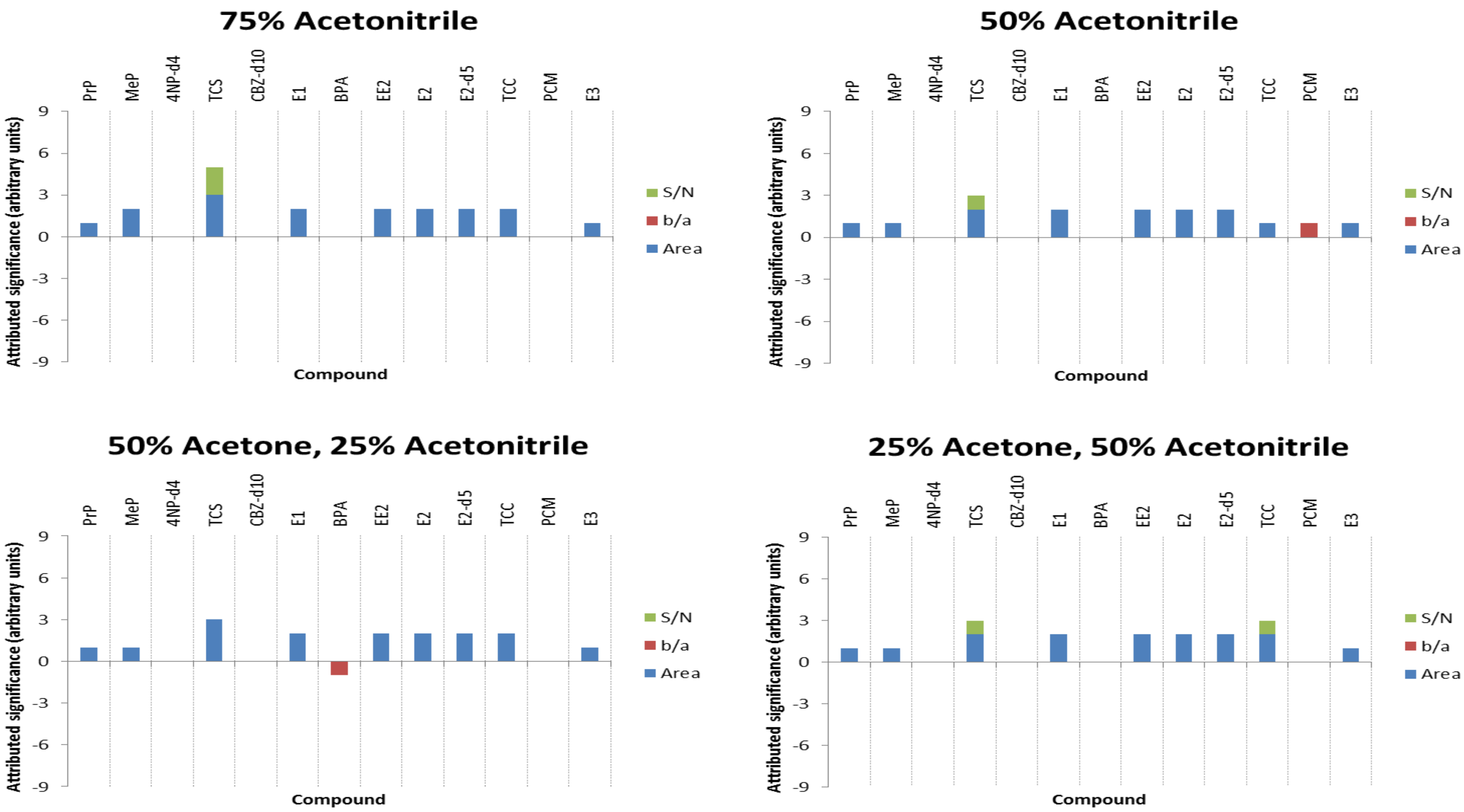

Figure 3.1 The effect of derivatizing solvent on a compounds signal-to-noise (S/N) ratio, peak shape (b/a) and area during UPLC-MS/MS analysis. Derivatizing solvent consisted part organic solvent (see figure titles) and part buffer (0.1 M $NaHCO_3$). The influence of the organic solvent part on a compounds' S/N, b/a or area are represented as a significant effect caused. Effects are arbitrarily quantified as 0, 1, 2 and 3, corresponding to the significant (ns, non-significant; *, p<0.05; **, p<0.01;*, p<0.001) change observed from the initial S/N, b/a and area of pre-optimization conditions. Positive values represent favourable changes whereas negative values unfavourable. Significance was calculated using one-way ANOVA (n=3) with Dunnett multiple comparison post-test.**

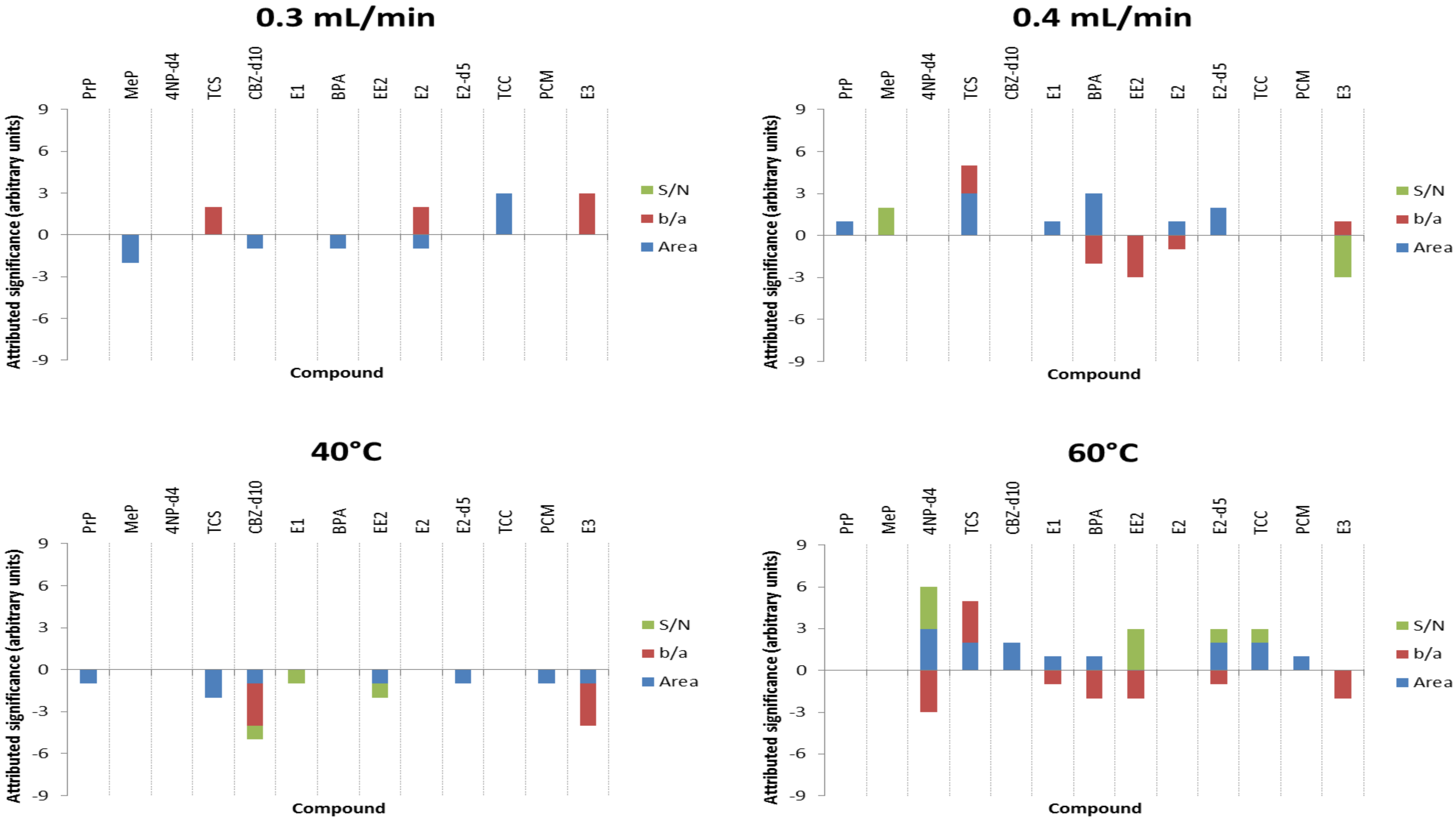

Figure 3.2 The effect of mobile phase flow rate (A) and column temperature (B) on a compounds signal-to-noise (S/N) ratio, peak shape (b/a) and area during UPLC-MS/MS analysis. The influence of mobile phase flow rate and column temperature on a compounds' S/N, b/a or area are represented as a significant effect caused. Effects are arbitrarily quantified as 0, 1, 2 and 3, corresponding to the significant (ns, non-significant; *, p<0.05; **, p<0.01;*, p<0.001) change observed from the initial S/N, b/a and area of pre-optimization conditions. Positive values represent favourable changes whereas negative values unfavourable. Significance was calculated using one-way ANOVA (n=3) with Dunnett multiple comparison post-test.**

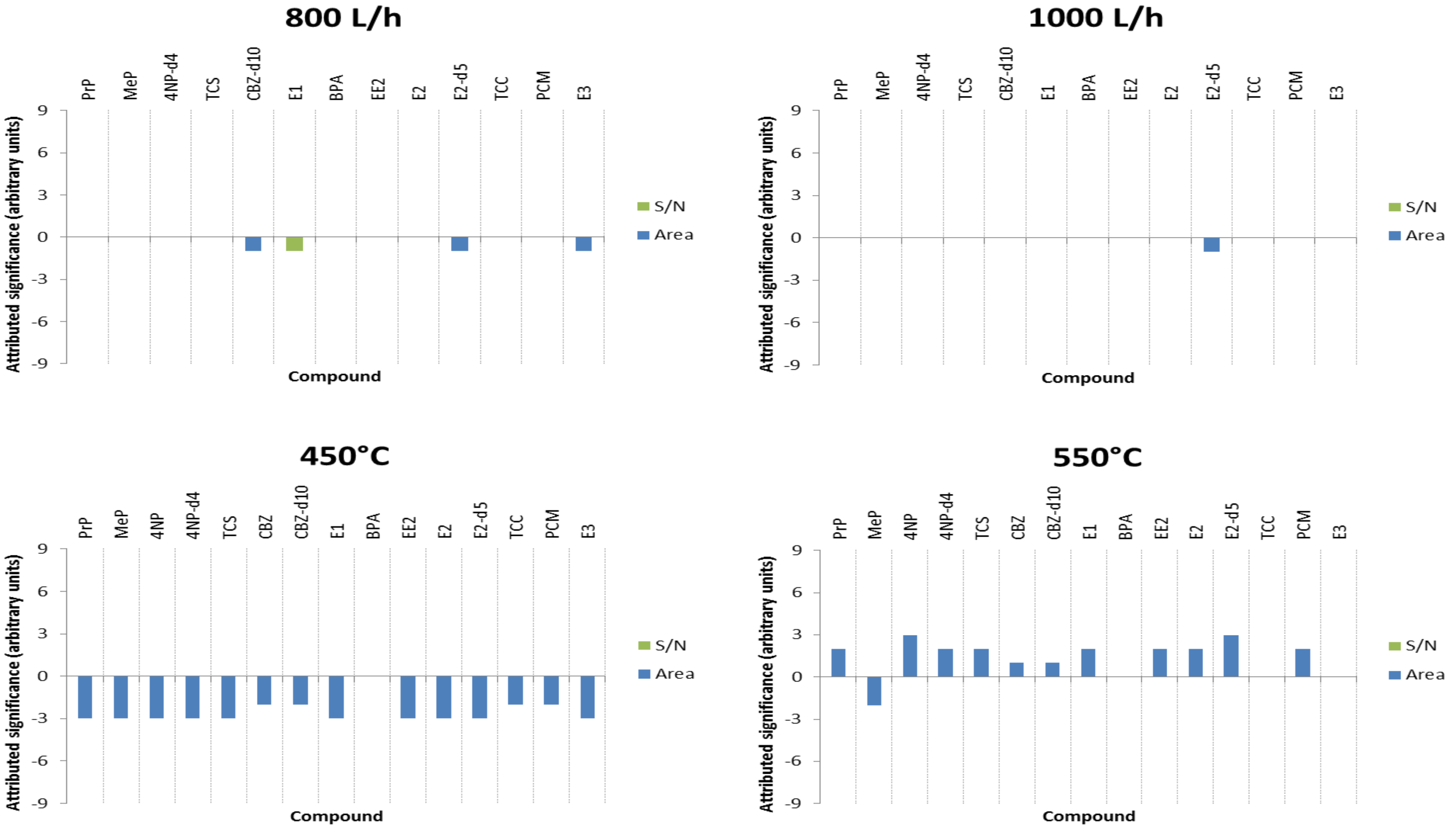

Figure 3.3 The effect of desolvation gas flow rate (A) and temperature (B) on a compounds signal-to-noise (S/N) ratio and area during UPLC-MS/MS analysis. The influence of desolvation gas flow rate and temperature on a compounds' S/N or area are represented as a significant effect caused. Effects are arbitrarily quantified as 0, 1, 2 and 3, corresponding to the significant (ns, non-significant; *, p<0.05; **, p<0.01;*, p<0.001) change observed from the initial S/N and area of pre-optimization conditions. Positive values represent favourable changes whereas negative values unfavourable. Significance was calculated using one-way ANOVA (n=3) with Dunnett multiple comparison post-test.**

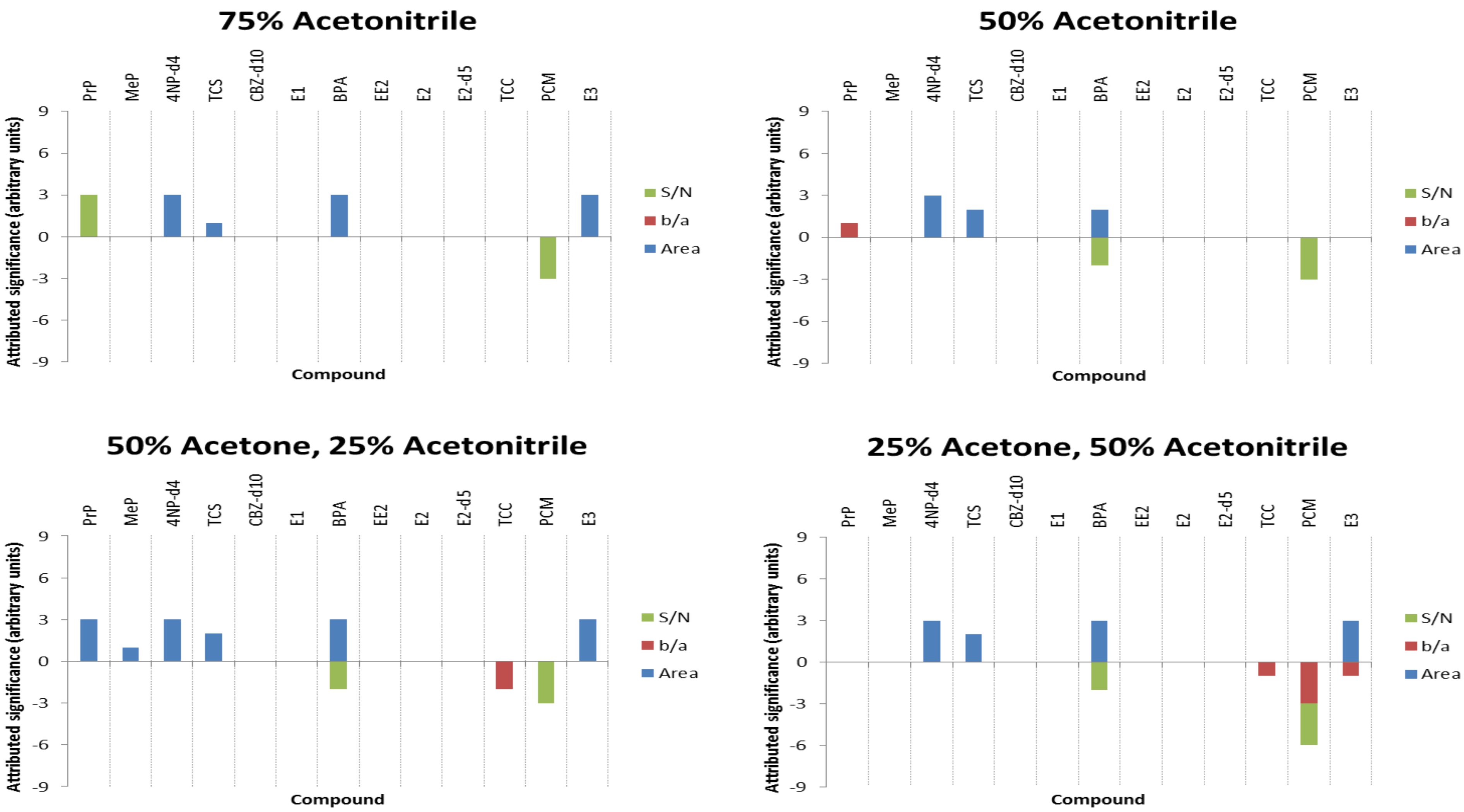

Figure 3.4 The effect of derivatizing solvent on a compounds signal-to-noise (S/N) ratio, peak shape (b/a) and area during UPC^2-MS/MS analysis. Derivatizing solvent consisted part organic solvent (see figure titles) and part buffer (0.1 M $NaHCO_3$). The influence of the organic solvent part on a compounds' S/N, b/a or area are represented as a significant effect caused. Effects are arbitrarily quantified as 0, 1, 2 and 3, corresponding to the significant (ns, non-significant; *, p<0.05; **, p<0.01;*, p<0.001) change observed from the initial S/N, b/a and area of pre-optimization conditions. Positive values represent favourable changes whereas negative values unfavourable. Significance was calculated using one-way ANOVA (n=3) with Dunnett multiple comparison post-test.**

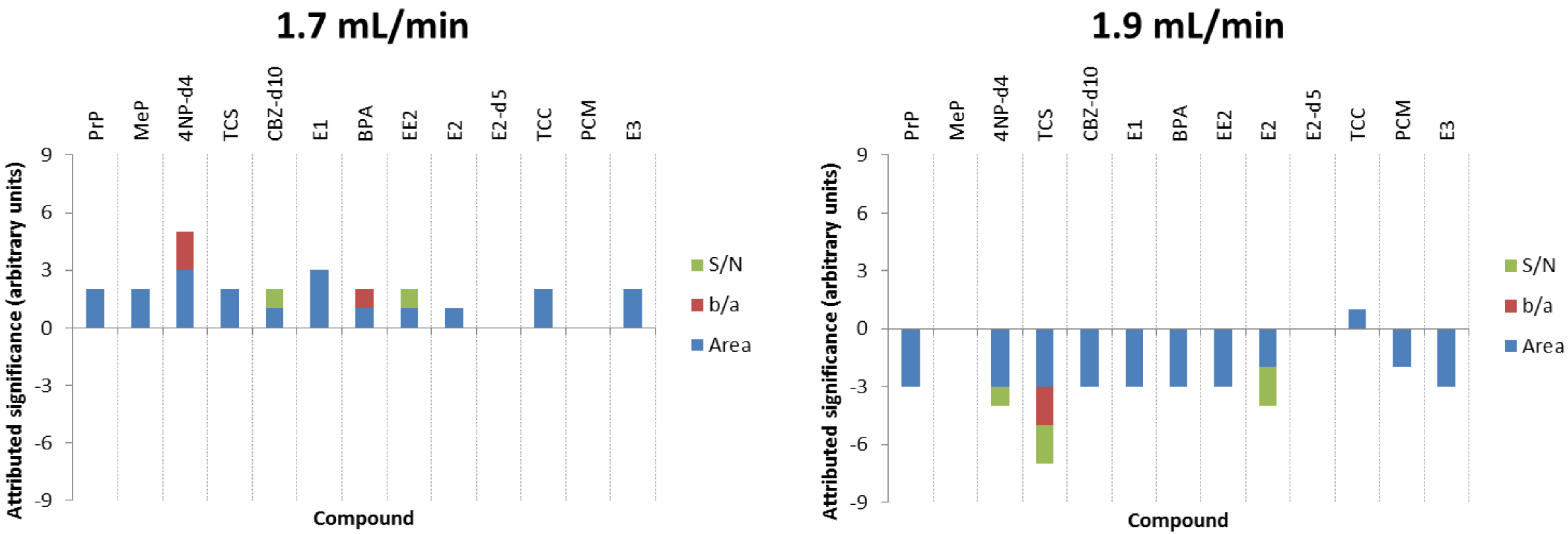

Figure 3.5 The effect of mobile phase flow rate on a compounds signal-to-noise (S/N) ratio, peak shape (b/a) and area during UPC^2-MS/MS analysis. The influence of mobile phase flow rate and column temperature on a compounds' S/N, b/a or area are represented as a significant effect caused. Effects are arbitrarily quantified as 0, 1, 2 and 3, corresponding to the significant (ns, non-significant; *, p<0.05; **, p<0.01;*, p<0.001) change observed from the initial S/N, b/a and area of pre-optimization conditions. Positive values represent favourable changes whereas negative values unfavourable. Significance was calculated using one-way ANOVA (n=3) with Dunnett multiple comparison post-test.**

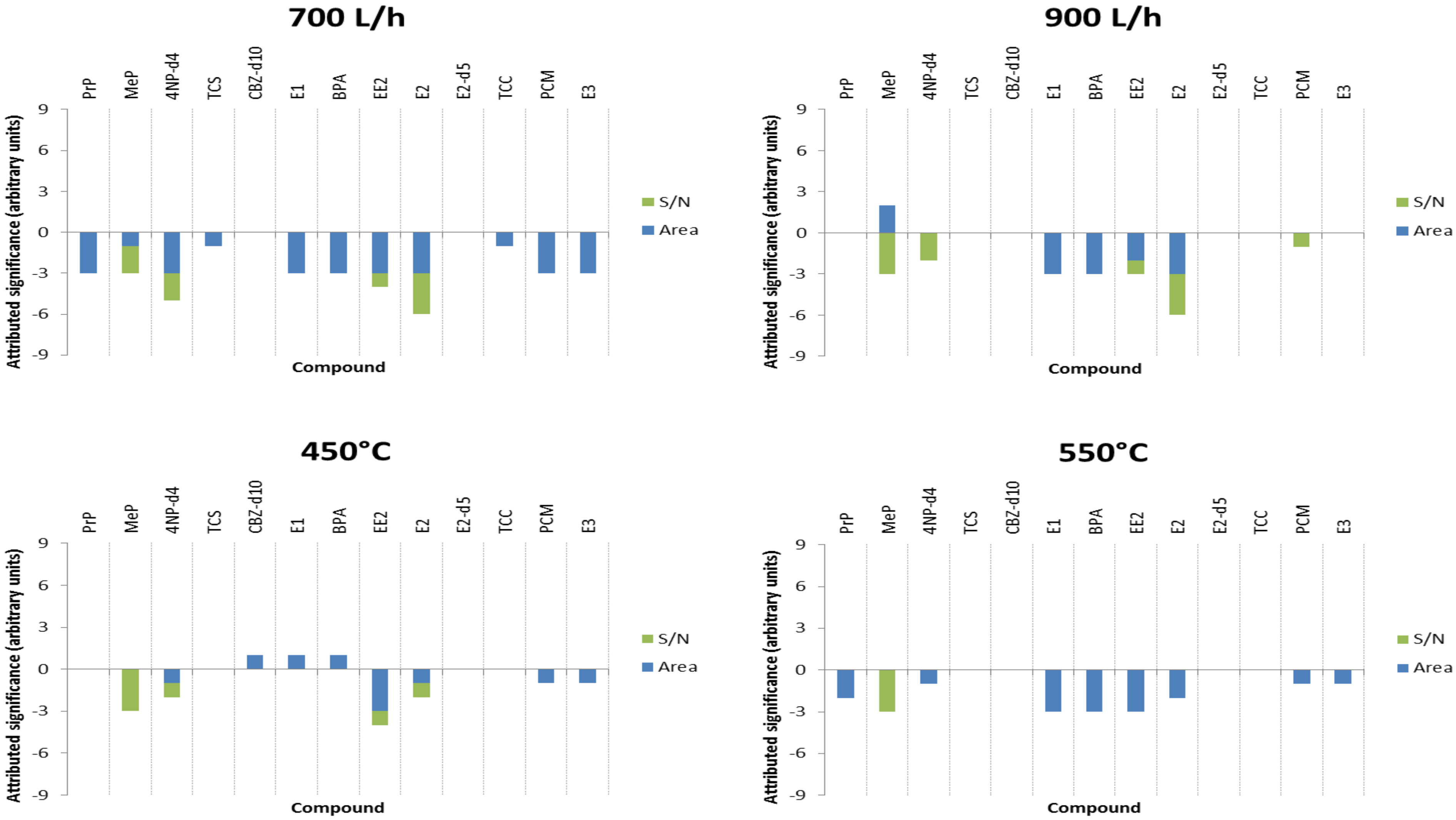

Figure 3.6 The effect of desolvation gas flow rate (A) and temperature (B) on a compounds signal-to-noise (S/N) ratio and area during UPC^2-MS/MS analysis. The influence of desolvation gas flow rate and temperature on a compounds' S/N or area are represented as a significant effect caused. Effects are arbitrarily quantified as 0, 1, 2 and 3, corresponding to the significant (ns, non-significant; *, p<0.05; **, p<0.01;*, p<0.001) change observed from the initial S/N and area of pre-optimization conditions. Positive values represent favourable changes whereas negative values unfavourable. Significance was calculated using one-way ANOVA (n=3) with Dunnett multiple comparison post-test.**

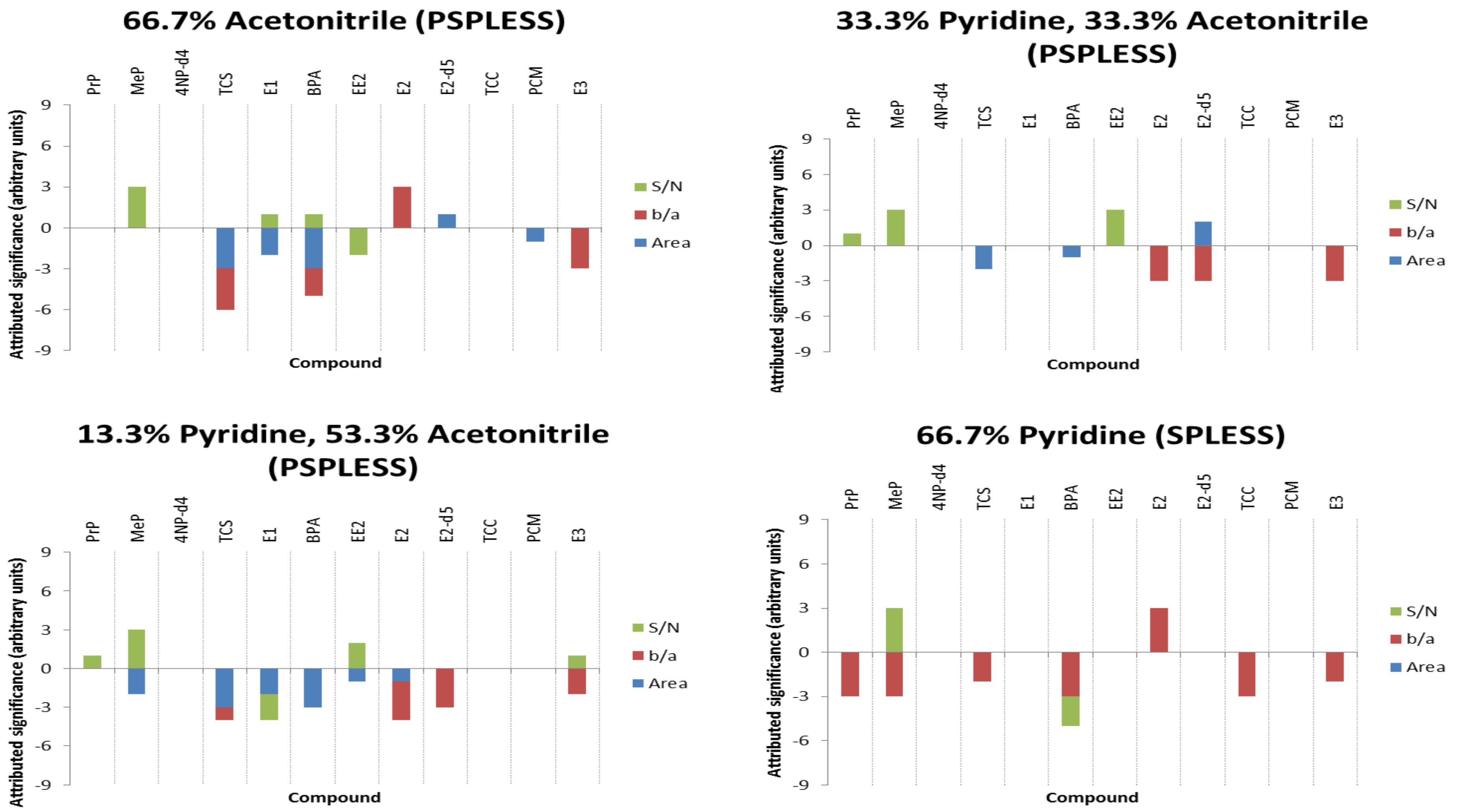

Figure 3.7 The effect of derivatizing solvent and injection mode on a compounds signal-to-noise (S/N) ratio, peak shape (b/a) and area during GC-MS/MS analysis. Derivatizing solvent consisted part organic solvent (see figure titles) and part BSTFA. The influence of the organic solvent part and injection mode (PSPLESS, pulsed splitless; SPLESS, splitless) on a compounds' S/N, b/a or area are represented as a significant effect caused. Effects are arbitrarily quantified as 0, 1, 2 and 3, corresponding to the significant (ns, non-significant; *, p<0.05; **, p<0.01;*, p<0.001) change observed from the initial S/N, b/a and area of pre-optimization conditions. Positive values represent favourable changes whereas negative values unfavourable. Significance was calculated using one-way ANOVA (n=3) with Dunnett multiple comparison post-test.**

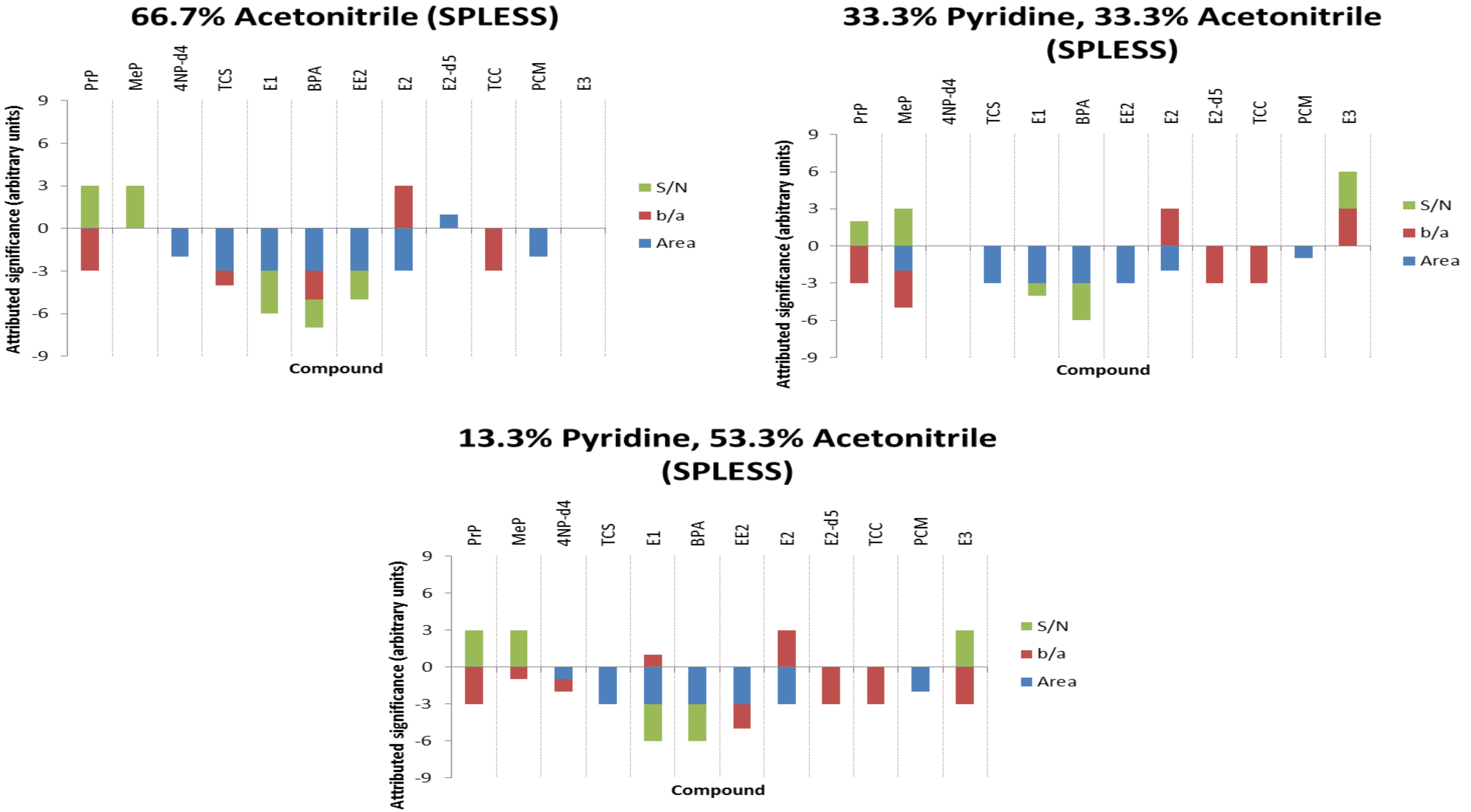

Figure 3.8 The effect of derivatizing solvent and injection mode on a compounds signal-to-noise (S/N) ratio, peak shape (b/a) and area during GC-MS/MS analysis. Derivatizing solvent consisted part organic solvent (see figure titles) and part BSTFA. The influence of the organic solvent part and injection mode (PSPLESS, pulsed splitless; SPLESS, splitless) on a compounds' S/N, b/a or area are represented as a significant effect caused. Effects are arbitrarily quantified as 0, 1, 2 and 3, corresponding to the significant (ns, non-significant; *, p<0.05; **, p<0.01;*, p<0.001) change observed from the initial S/N, b/a and area of pre-optimization conditions. Positive values represent favourable changes whereas negative values unfavourable. Significance was calculated using one-way ANOVA (n=3) with Dunnett multiple comparison post-test.**

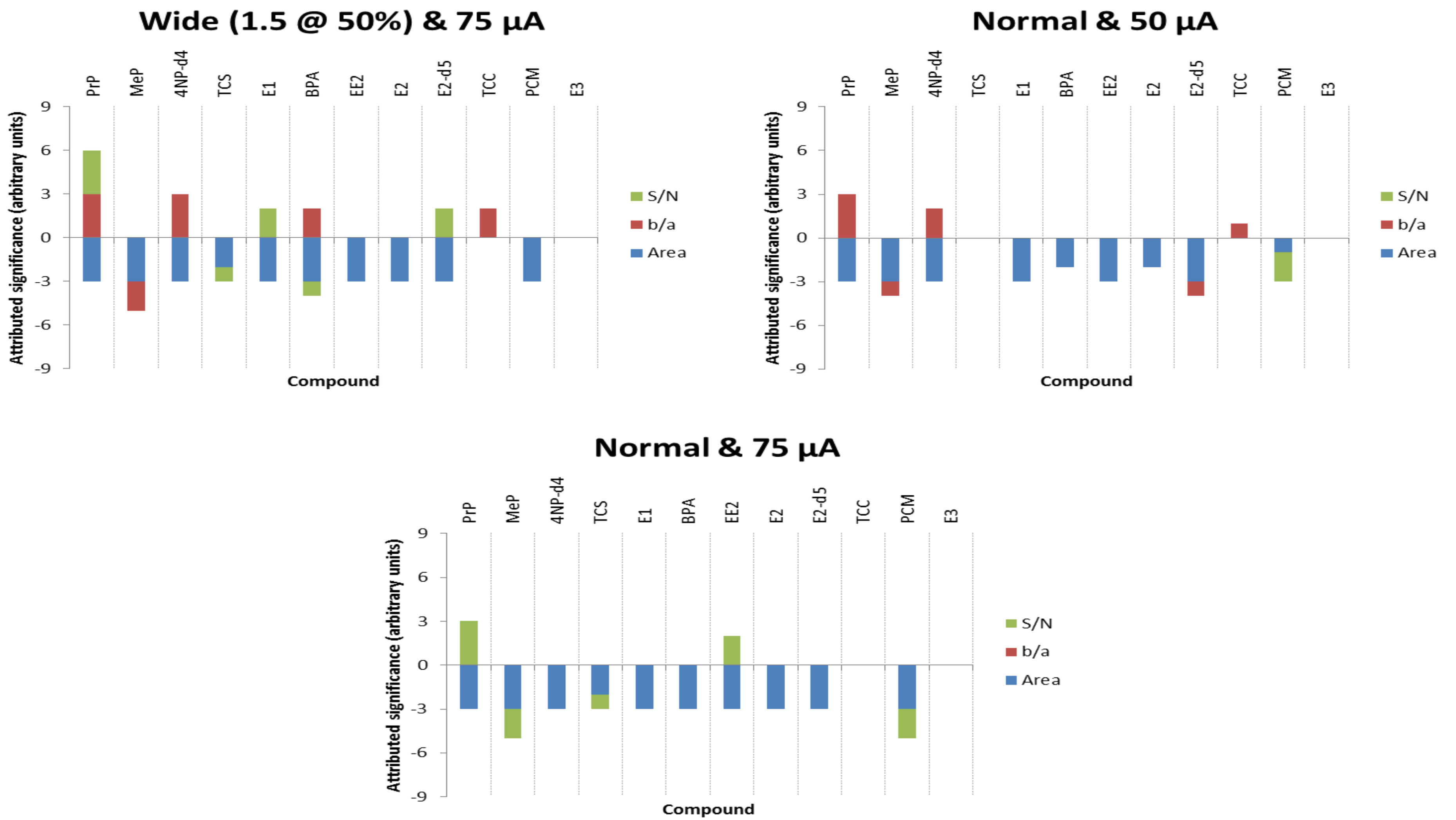

Figure 3.9 The effect of Q1 resolution and emission current on a compounds signal-to-noise (S/N) ratio, peak shape (b/a) and area during GC-MS/MS analysis. The influence of the Q1 resolution and emission current on a compounds' S/N, b/a or area are represented as a significant effect caused. Effects are arbitrarily quantified as 0, 1, 2 and 3, corresponding to the significant (ns, non-significant; *, p<0.05; **, p<0.01;*, p<0.001) change observed from the initial S/N, b/a and area of pre-optimization conditions. Positive values represent favourable changes whereas negative values unfavourable. Significance was calculated using one-way ANOVA (n=3) with Dunnett multiple comparison post-test.**

3.2.3 Analytical Capabilities of each instrument

To assess the performance of each optimized method on the three different instruments, a mixture of 250 ppb was prepared and a dilution series created to find the limit of detection (LOD), limit of quantification (LOQ) and upper limit of quantification (ULOQ). Together LOQ and ULOQ are regarded as the linear region of quantification for which the R^2 value is an indication of linearity. The limits for the UPLC- and UPC^2-MS/MS instruments were tested on the UPLC-MS/MS instrument and extrapolated to the UPC^2-MS/MS instrument. Although this extrapolation is not entirely correct seeing that ionization efficiency is influenced by the composition of the mobile phase, which differs between these systems, the results obtained from the UPLC-MS/MS data provided insight into the possible detection and quantification limits obtainable with the mass spectrometer in question. The analytical capabilities of UPLC/UPC^2-MS/MS are given in Table 3.7 and that of GC in Table 3.8.

From the tabulated results it is evident that both UPLC/UPC^2-MS/MS and GC-MS/MS will be sufficiently sensitive in determining sub ppb concentrations with exceptional linear signal strength over the specified linear range. However, the UPLC/UPC^2-MS/MS instrument was more sensitive than GC-MS/MS with an extended quantification range for all compounds, offering ultra-low level detection and quantification.

Table 3.7 UPLC and UPC^2-MS/MS analytical capabilities. LOD, limit of detection; LOQ, limit of quantification; ULOQ, upper limit of quantification; ND, not detected.

Compound	LOD (ppb)	LOQ (ppb)	ULOQ (ppb)	R^2
PrP	0.005	0.05	250	0.998
MeP	<0.001	0.001	5	0.991
TCS	<0.001	0.05	100	0.997
E_1	0.05	0.1	100	1
BPA	<0.001	0.25	100	0.998
EE_2	0.05	0.25	100	1
E_2	0.05	0.1	100	1
PCM	0.05	0.05	100	0.999
E_3	0.05	0.05	250	0.999
TCC	ND	ND	ND	ND

Table 3.8 GC-MS/MS analytical capabilities. LOD, limit of detection; LOQ, limit of quantification; ULOQ, upper limit of quantification; ND, not detected.

Compound	LOD (ppb)	LOQ (ppb)	ULOQ (ppb)	R^2
PrP	0.1	0.25	100	1
MeP	0.25	0.5	100	1
TCS	0.5	0.5	100	0.999
E_1	0.1	5	100	0.998
BPA	1	5	100	0.999
EE_2	0.5	1	100	0.998
E_2	0.25	1	100	0.999
PCM	ND	ND	ND	ND
E_3	0.5	5	100	0.995
TCC	ND	ND	ND	ND

3.2.4 SPE cartridge selection for the pre-concentration of EDCs

By far the most commonly used sample preparation methods used for pre-concentrating compounds from liquid matrices are liquid-liquid- and solid-phase extraction (SPE). However of these, SPE is probably the most straight-forward and preferred sample preparation method available if the cost of SPE cartridges is negligible. In SPE, compounds are removed from the matrix by passing the liquid matrix through a SPE cartridge containing a stationary phase that selects for the desired compounds. The concentrated compounds are then eluted from the column by applying a solvent with a higher affinity for the stationary phase. Various SPE cartridges are currently available on the market that is able to select for a wide variety of compounds with different chemical properties.

To pre-concentrate the 9 EDCs from water and waste water matrices, two SPE cartridges were under consideration: a strong lipophilic C18 SPE cartridge (Supelclean ENVI-18) and a ‗dual function' SPE cartridge (Supel-select HLB) that selects for compounds with both hydrophilic and lipophilic properties. A screen was performed on each to determine which would yield the best result – i.e. retaining the highest possible number of selected EDC at the highest possible eluent strength. This provides both selectivity and ensures that eluents with a weaker strength can be used to remove unwanted compounds found in complex matrices. In the screen compounds were loaded onto SPE cartridges and varying concentration of methanol (MeOH), at three different pH's, used to elute the compounds from the cartridges.

The varying concentration range provides insight into the SPE elution profile while at the same time suggesting the optimum pH for each compound. Results are shown in **Error! Reference source not found.** and indicate that the Supel-select HLB cartridges ind the selected compounds stronger to the stationary phase, allowing the use of a stronger wash step to purge the cartridge from unwanted weakly bound matrix constituents. Furthermore, 60% MeOH seems to be a good wash solvent as 9/10 compounds are still bound to the stationary phase after use, the only exception being PCM. From this data preliminary studies were performed that investigated the possible recoveries obtainable under different acidification conditions. Results are shown in **Error! eference source not found.**.

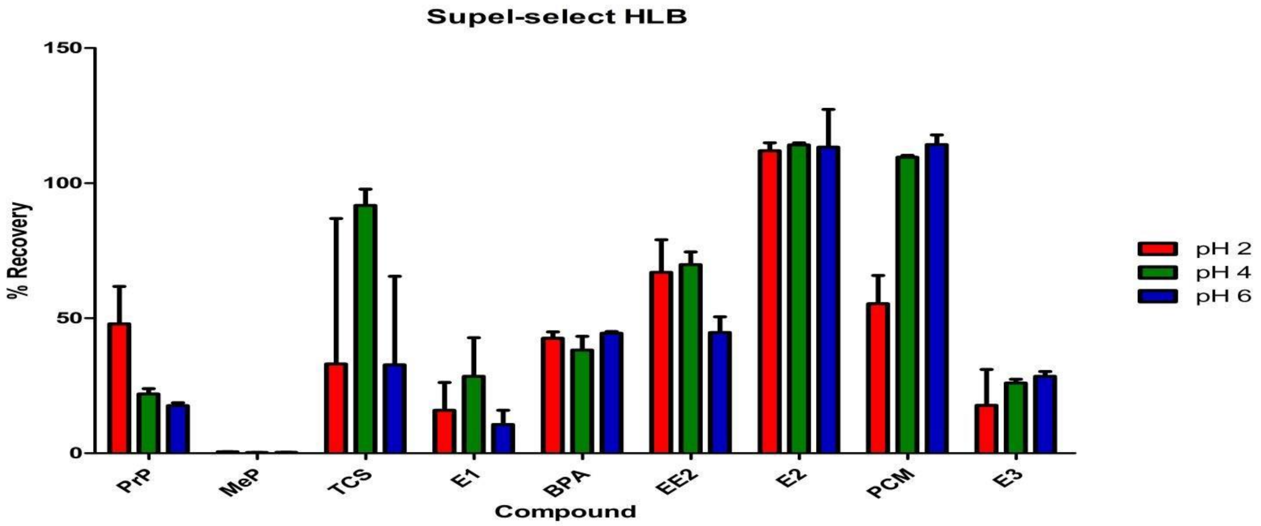

Figure 3.10Preliminary recoveries obtained for each of the 9 compounds extracted from different acidified water matrices using Supel-select HLB cartridges conditioned with and aqueous phase similar to that of the sample. Formic acid (1%), 10 mM ammonium formate and 10 mM ammonium acetate were used to acidify equilibration solutions, samples and wash steps. Two-way ANOVA followed by a Bonferroni post-test were used to test for significant differences. No significance was observed

Statistical analysis of the possible recoveries obtainable under different acidification conditions indicated neither that 1% formic acid nor 10 mM ammonium formate or 10 mM ammonium acetate yield recoveries more desirable than the other. From this data estimated recoveries were determined under 1% formic acidified equilibration solutions, samples and wash solvents. Both water and SWW were investigated as to determine expected recoveries from these matrices. The filtering of samples was also investigated to simulate removal of excess debris that might clog SPE cartridges. Estimated recoveries are given in Table 3.9.

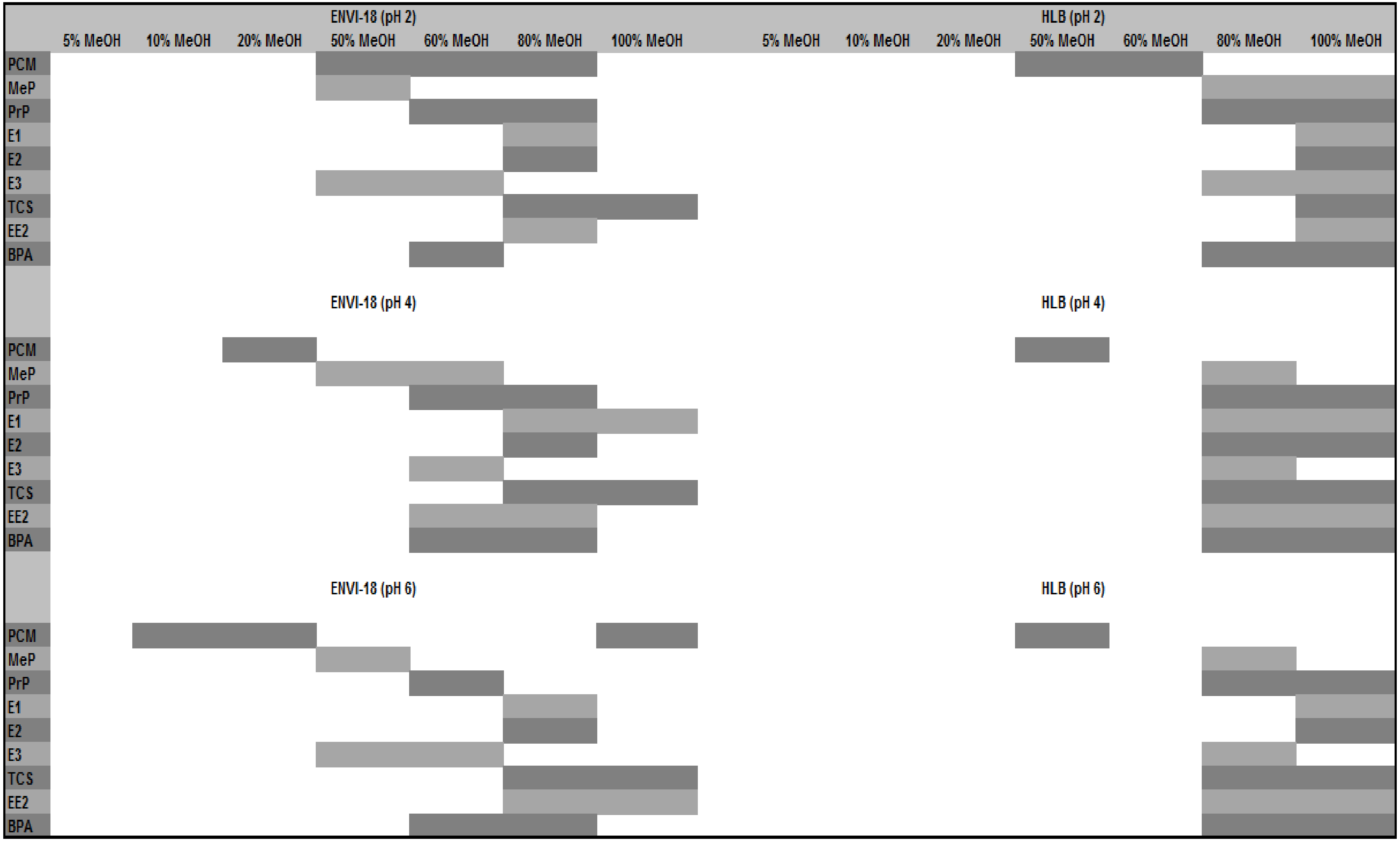

Figure 3.11 Heatmap showing the elution profile of each compound on the two different SPE cartridges under different pH conditions. The map only indicate the presence of a compound at the a specific elution step and by no means infer quantity.

Table 3.9 Estimated percentage recovery for the different compound in two matrices and under two pre-treatment conditions. Compounds were extracted from a HLB cartridge (preconditioned to pH 2) preloaded with unfiltered or filtered water or synthetic waste water (SWW). Values represent the mean ± SE (n= 3). Statistical significance between unfiltered water, filtered water, unfiltered SWW and filtered SWW was determined with a two-way ANOVA followed by a Bonferroni post-test.

Compound	Unfiltered water	Filtered water	Unfiltered SWW	Filtered SWW
PrP	87.6 ± 1.87^{a}	87.2 ± 2.01^{ab}	1146 ± 136^{c}	3047 ± 318
MeP	176 ± 10.1^{a}	65.8 ± 11.7^{ab}	1086 ± 135^{c}	988 ± 211^{c}
TCS	87.2 ± 18.1^{a}	145 ± 26.1^{ab}	376 ± 12.1^{abc}	2658 ± 831
E_1	72.6 ± 1.87^{a}	75.3 ± 9.85^{ab}	81.4 ± 5.84^{abc}	96.3 ± 17.0^{abc}
BPA	98.6 ± 27^{a}	206 ± 4.33^{ab}	134 ± 9.34^{abc}	247 ± 24.9^{abc}
EE_2	66.9 ± 6.44^{a}	246 ± 14.3^{ab}	215 ± 39.0^{abc}	86.6 ± 10.7^{abc}
E_2	73.9 ± 0.968^{a}	67.0 ± 4.36^{ab}	74.5 ± 3.60^{abc}	109 ± 34.0^{abc}
PCM	2.63 ± 0.275^{a}	6.93 ± 5.54^{ab}	20.0 ± 3.18^{abc}	0.041 ± 0.003^{abc}
E_3	193 ± 19.3^{a}	39.9 ± 10.0^{ab}	785 ± 28.8^{c}	1081 ± 190^{c}
TCC	159 ± 28.8^{a}	123 ± 44.6^{ab}	0.926 ± 0.236^{abc}	28.3 ± 26.3^{abc}

All compounds from unfiltered water showed good recovery, with the exception of PCM showing a very low recovery. Furthermore, filtering of water seemed to have little effect on recovery compared to that of unfiltered water. Recoveries from unfiltered and filtered SWW showed unusually high recoveries for PrP, MeP, TCS and E_3. Finally, like the filtering of water, the filtering of SWW also increased the recoveries of certain compounds such as PrP and TCS.

3.2.5 Method validation

After extensive optimization of all the instruments being used and the EDC pre-concentration protocol, the best performing instrument and optimized EDC pre-concentration protocol needed to be validated as a whole. Seeing that small variations in method performance between UPLC- and UPC^2-MS/MS were not investigated and that the UPC^2 instrument is new to the market, it was decided to do the method validation procedure on the UPC^2-MS/MS. Two recent papers by Kruve *et al.*(43, 44) were used as guidelines for the method validation. The results from the validation procedure are shown in Table 3.10 - Table 3.12 and **Error! Reference source not found.**.

Table 3.10 Comprehensive method validation data: Limit of detection (LOD, n = 3), limit of quantification (LOQ, n = 3), upper limit of quantification (ULOQ, n = 3), signal linearity (R^2), instrumental accuracy (% RSD, n = 4), precision (% RSD, n = 6) and percentage stability (mean ± SE, n = 4). Statistical analysis on the % stability of compounds was performed with a one-way ANOVA, followed by a dunnett post-test. Significance is indicated by *, ** and * representing $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. TLTQ, Too low to quantify.**

Compound	LOD	LOQ	ULOQ	R^2	Instrumental accuracy % RSD (ppb)	Precision % RSD (ppb)			% Stability
	pg on column	pg on column	pg on column			Solvent	Water	SWW	
PrP	0.05	0.1	20	0.986	6.47 (1)	2.01 (1)	1.76 (1)	0.627 (1)	103 ± 3.62
MeP	0.005	0.01	1	0.752	18.8 (0.1)	17.3 (0.1)	6.66 (0.1)	17.8 (0.1)	112 ± 6.54
TCS	0.05	0.05	20	0.990	11.3 (1)	4.25 (1)	4.66 (1)	5.83 (1)	99.3 ± 8.25
E_1	0.05	0.05	20	0.997	17.1 (1)	2.01 (1)	4.38 (1)	5.09 (1)	76.6 ± 0.327***
BPA	0.125	0.125	50	0.995	5.86 (2.5)	6.56 (2.5)	4.43 (2.5)	6.38 (2.5)	96.5 ± 0.566
EE_2	0.125	0.125	50	0.996	1.69 (2.5)	2.76 (2.5)	9.89 (2.5)	9.14 (2.5)	99.2 ± 0.669
E_2	0.05	0.05	20	0.998	1.30 (1)	2.95 (1)	2.60 (1)	2.71 (1)	101 ± 0.817
PCM	0.125	0.125	50	0.993	1.57 (2.5)	4.33 (2.5)	3.63 (2.5)	TLTQ (2.5)	102 ± 1.85
E_3	0.05	0.1	20	0.995	0.626 (1)	3.82 (1)	8.91 (1)	2.31 (1)	130 ± 0.749***
TCC	0.1	0.1	20	0.893	4.05 (1)	19.5 (1)	77.3 (1)	16.8 (1)	136 ± 7.35***

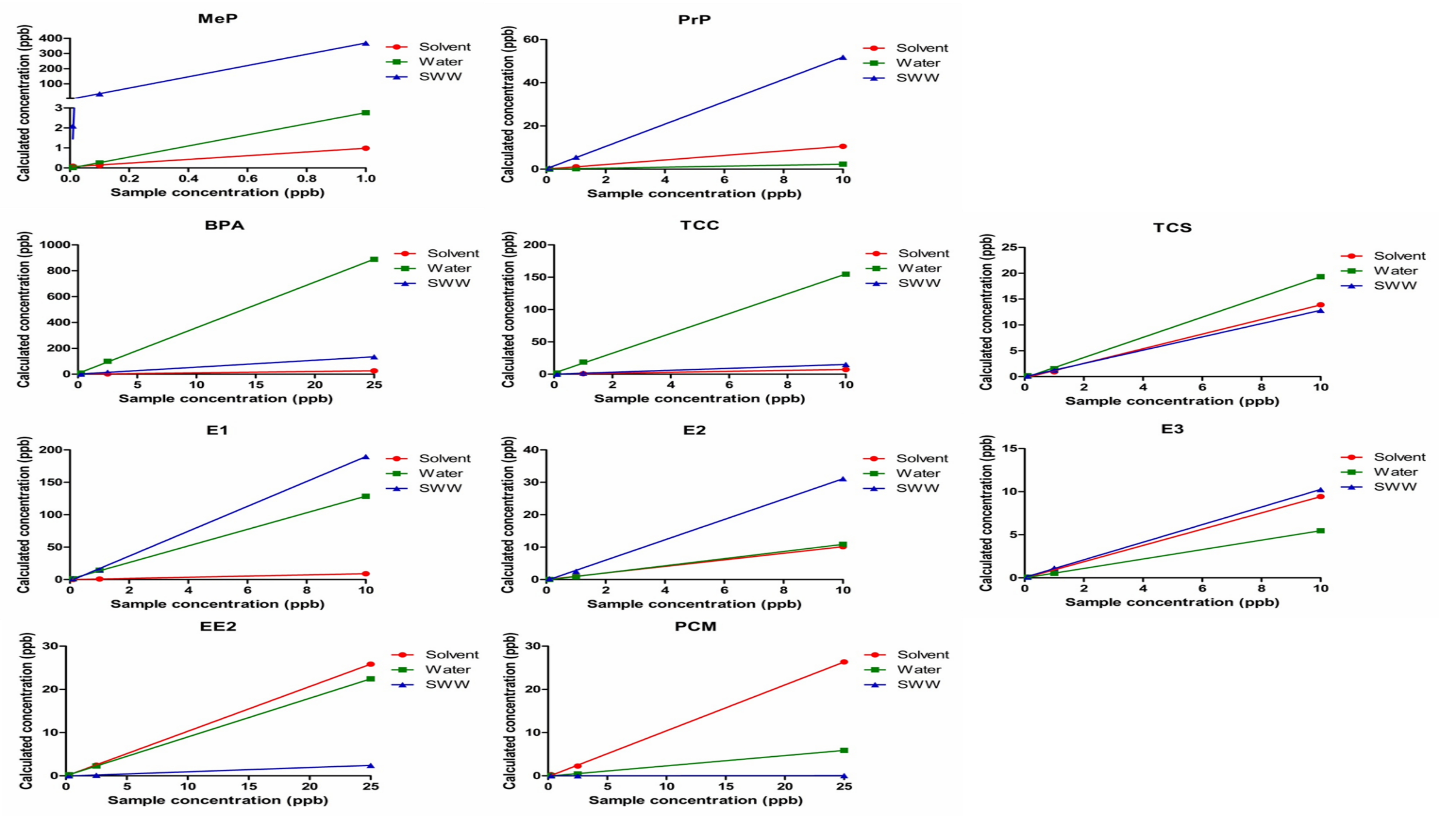

Figure 3.12 The matrix effect of matrix constituents on each compound. Where the water and SWW matrices regression lines appear above that of the solvent, ionization is enhanced, whereas below the solvent line indicates ionization suppression. Linear regression lines were drawn with the statistical package GraphPad Prism and departure from linearity tested for with runs test. The matrix effect observed under solvent conditions for BPA, TCC and E_1 are clearly wrong as solvent conditions would yield better conditions than both water and SWW. It is possible that these compounds did not dissolve properly when stocks were created.

Table 3.11 Comprehensive method validation data (continued): Process efficiency (%, n = 3) at a low (C1), medium (C2) and high (C3) SPE cartridge load.

	Investigator 1						Investigator 2					
Compound	Water			SWW			Water			SWW		
	C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
MeP	3.40	37.0	16.3	552	1.50×10^3	2.23×10^3	11.3	138	56.1	7.33×10^3	4.96×10^4	7.84×10^4
PrP	5.32	21.2	8.94	40.5	340	68.1	10.3	50.2	25.9	500	1.70×10^3	679
TCS	38.3	172	109	15.7	66.5	29.8	133	72.2	54.8	192	246	183
TCC	109	1.47E+03	194	62.6	147	81.9	1.08×10^3	1.75×10^3	1.42×10^3	90.3	154	347
E_1	2.25×10^3	2.46×10^3	1.05×10^3	1.93×10^3	2.07×10^3	1.33×10^3	517	509	333	2.11×10^3	1.07×10^3	1.27×10^3
E_2	85.9	94.9	108	258	192	236	60.5	44.0	114	311	274	201
E3	99.8	27.5	31.2	64.7	119	92.6	28.7	288	42.7	97.5	165	163
EE_2	76.9	117	130	5.2	6.94	7.36	47.7	65.0	91.5	2.11	25.4	16.4
BPA	1.59×10^3	1.98×10^3	205	319	599	382	2.48×10^3	4.17×10^3	2.26×10^3	170	164	45.9
PCM	22.8	14.3	2.15	0.080	0.068	0.00	20.5	49.2	9.20	0.00	85.3	25.7

Table 3.12 Comprehensive method validation data (continued): Recovery (%, n = 3) at low (C1), medium (C2) and high (C3) SPE cartridge load. ULOQ, upper limit of quantification.

	Investigator 1						Investigator 2					
Compound	Water			SWW			Water			SWW		
	C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
MeP	13.0	23.5	6.21	2.42	82.8	15.3	10.2	74.2	94.3	4.20	53.4	65.6
PrP	20.8	44.3	19.0	14.1	64.1	63.8	34.9	203	119	23.0	128	69.8
TCS	0.0	94.6	56.4	33.7	48.5	39.2	0.00	105	56.3	38.6	99.9	132
TCC	11.1	58.6	8.37	42.7	63.3	36.2	32.5	139	40.3	67.0	70.3	153
E_1	95.0	146	94.5	0.00	105	115	34.1	68.6	16.5	0.00	4.20	15.3
E_2	0.00	95.5	114	>ULOQ	106	78.8	0.00	60.1	114	>ULOQ	107	79.6
E_3	228	43.9	89.5	55.9	106	90.4	65.8	909	93.2	74.0	170	159
EE_2	54.8	67.3	72.7	0.00	35.4	37.6	27.4	36.2	51.0	0.0	360	24.6
BPA	17.5	50.2	36.6	42.1	52.2	48.1	27.3	53.9	35.7	13.1	10.2	4.53
PCM	107	24.6	3.86	0.00	0.00	0.02	115	99.0	17.9	0.00	2.72	1.52

Table 3.10 shows similar sensitivities to those predicted for the different compounds in the preliminary study, with all three investigated concentration (C1, C2 and C3) for all compounds falling within the predicted linear region of quantification. Furthermore, E_1, E_2, EE_2 and BPA showed lower LOQs than originally determined by UPLC-MS/MS. However, PrP, MeP and E_3 showed higher LOQs with decreased signal linearity for MeP. Finally, TCC could be detected, its linear range and linearity of signal determined – not previously possible. Method accuracy experiments showed that the measured values for all compounds were in close relation to their true value, while precision experiments indicated that solvent and both water and SWW prepared sample concentration could be detected with high precision. Percentage stability reports suggest that most compounds stay stable in the final derivatization solution for at least 18 hours. Only TCC, E_1 and E_3 show deviations from stability.

Data from **Error! Reference source not found.** suggest that water has an onization enhancement effect on MeP, TCS, TCC, BPA and E_1, suppression effect on PrP, E_3, EE_2 and PCM, while having little to no effect on the ionization of E_2. In contrast, SWW shows ionization enhancement for MeP, PrP, BPA, E_1 and E_2, a suppression effect on EE_2 and PCM, while having no or little effect on the ionization of TCS, TCC and E_3.

Process efficiency and recovery was highly variable for most compounds between investigators and between the different matrices (Table 3.11 and Table 3.12). TCS, TCC, E_1, E_2 and E_3 showed some degree of agreement between investigators and between matrices for process efficiency. Furthermore, compounds such as MeP, PrP, TCC, E_1 and BPA showed unachievably high process efficiencies and E_2 unachievably high recoveries. PCM in contrast to unachievably high process efficiencies and recoveries showed a very low to no process efficiency and recovery.

3.3 Discussion

In the present study the aim was to develop analytical methods that would enable the pre-concentration, detection and quantification of known EDCs in the environment, as tool to evaluate the JTED Ecomachine. Three highly sensitive analytical instruments were identified to detect and quantify the pre-concentrated EDCs from the environment, namely a UPLC-, UPC^2- and GC-MS/MS system. Methods were developed on each instrument and optimized by changing variables such injection solvent, column temperature, eluent flow rate, and desolvation gas temperature and flow rate, before selecting the optimal conditions. The optimized instrumental methods were then evaluated for their sensitivity.

Together with their feasibility, i.e. how many of the selected EDCs can be detected and quantified, and economic friendliness one instrument was selected for the final method validation. Next, a SPE protocol was developed that would enable the pre-concentration of the compounds of interest from environmental samples. Here, two SPE cartridges with different stationary phase chemistries were considered. In addition, an optimum extraction pH (2, 4, or 6) was examined. Second to last, a preliminary recovery study for the pre-concentration protocol was performed to determine its feasibility before extensive method validation. Finally, an extensive method validation study was carried out to evaluate the pre-concentration, identification and quantification protocol as whole.

Method development was initiated by first setting up the relevant SRMs or MRMs on each instrument. This required the direct injection of each derivatized compound on each instrument separately. Table 3.2 shows the optimized MRMs for each compound set up on both the UPLC- and UPC^2-MS/MS. For UPLC- and UPC^2-MS/MS the base peak observed for all compounds except CBZ-d_{10} and TCC was that op the product ion at m/z 171. The main secondary product ion for these compounds was found to be at m/z 156, with the exception of BPA whose secondary ion was selected to be at m/z 170. These product ions corresponds to previous studies utilizing DNCl as derivatizing agent (41, 358, 359). The product ion for BPA has not been reported before using an ESI mass spectrometer. TCC and CBZ-d_{10} did not show these product ions as these compounds do not get derivatized with DNCl under the conditions prescribed. The product ions m/z 160 (base peak) and m/z 126 observed for TCC have been report by Hancock *et al.* (360) and Jongmun *et al.* (361). The product ions found for CBZ-d_{10} corresponded to an m/z at 202 and 204 (base peak), which is 10[^{2}H] heavier than the reported m/z 192 and m/z 194 reported for CBZ (362, 363). Finally, each compound was injected on the relevant columns and the retention time on each column noted in Table 3.1. From the results in Table 3.1 it is evident that sufficient separation was achieved for all compounds on all UPC^2 columns. However, TCC eluted at nearly the same retention time as that of E_3 and MeP on the UPLC column. Both E_3 and MeP MRM scans were performed in positive mode and therefore greatly influenced the sensitivity of the method for TCC. This is evident from Table 3.7 showing that TCC could not be detected when a preliminary sensitivity study was performed. For GC-MS/MS analysis Table 3.3 shows the relevant precursor and product ions as well as each compound‘s retention time on the column. The different precursors or product ion were matched to that found in literature (364–371). Like the UPC^2-MS/MS, sufficient separation of compounds could also be achieved on the GC-MS/MS.

After method development, certain variables were investigated to improve the peak area, peak shape and S/N. The optimized conditions are laid out in Table 3.4, Table 3.5 and Table 3.6. According to *Kruve et al.*(43), of the parameters being investigated on the UPLC, injection solvent composition/derivatizing solvent have the highest likelihood to have an effect on parameters such as peak shape, area and S/N. However, the current study showed that column temperature played a bigger role (**Error! Reference source ot found.**, **Error! Reference source not found.** and **Error! Reference source not found.**). Nevertheless, injection solvent composition significantly affected the peak area of all compounds investigated. This was also the case for desolvation gas temperature. Next to column temperature, desolvation flow rate had the greatest effect on peak shape, while all variables contributed similarly to the effect on S/N. A possible explanation for the larger role column temperature played in results could be explained by the temperature at which derivatization is normally carried out. The optimal heat conditions resembling that of the derivatization conditions could help influent compounds to be at the correct temperature for all to be at their derivatized masses before injection into the mass spectrometer. On the UPC^2-MS/MS, injection solvent composition affected the parameters the least, while all other variables played an equally important role on parameter improvements/impairments (**Error! Reference source not found.**, **Error! Reference source not found.** and **Error! erence source not found.**). The smaller effect injection solvent composition played suggests that the UPC^2 instrument may be more compatible with a diverse range of injection solvents. Lastly, flow rate showed to be the most effective in improving the overall performance of the method. In contrast to both UPLC- and UPC^2-MS/MS analysis, injections solvent composition and split mode on GC-MS/MS displayed the greatest effect on parameters (**Error! Reference source not found.**, **Error! Reference source not und.** and **Error! Reference source not found.**). Although it is impossible to pinpoint the main contributor to parameter improvements between solvent composition and split mode without testing the conditions separately, pulsed splitless injections have been shown to improve sample transfer and enable trace level analysis (372–375).

Before samples could be analysed using either developed instrumental method, a preliminary sensitivity study needed to be done to determine which system – UPLC/UPC^2 would yield the best results in future. This include being able to optimize the EDC pre-concentration protocol. Therefore, a dilution series was prepared consisting of a mixture of all the compounds under investigations. The dilution series was injected on the UPLC- and GC-MS/MS and preliminary LODs, LOQs, ULOQs and linearity‘s for the different compounds calculated. Results are shown in Table 3.7 and Table 3.8. The obtained results suggested that the UPLC/UPC^2-MS/MS instruments have higher sensitivities than the GC-MS/MS instrument and therefore latter was excluded from further investigations and the final extensive validation. While in the process of deciding which of the two instruments – UPLC or UPC^2 to use for the optimization of EDC extraction protocol from environmental matrices, the then used UPC^2 2-EP column failed and no other columns were available for use. This forced the use of the UPLC-MS/MS as instrument of choice for the use of optimizing the EDC pre-concentration protocol.

During the first trials of constructing an EDC pre-concentration protocol, the Supelclean ENVI-18 – a C18 cartridge, and a Supel-select HLB – a hydrophilic and lipophilic cartridge, were under investigation. Extractions were carried out on each and results showed that the HLB cartridge could retain compounds at a higher percentage MeOH wash step than the ENVI-18 (**Error! Reference source not found.**). Most EDC nvestigated in this study are hydrophobic in nature. However, many of the EDCs contain hydroxyl groups and therefore will interact better with a stationary phase also containing polar groups. In the final step of constructing the EDC pre-concentration protocol the pH at which compounds would be optimally retained on the HLB cartridge was investigated. Here different organic acids were used to acidify both the equilibration solvent as well as the sample. **Error! Reference source not found.** shows the preliminary recoveries under ifferent sample and equilibration solvent conditions. Statistical analysis revealed no noteworthy differences between the different acidification steps. However, extensive work by Baker and Kasprzyk-Hordern suggest acidifying samples to a pH of two for pharmaceuticals and illicit drugs in surface and waste water (376). From this suggestion the studies that followed used a pH of two. Finally, a more comprehensive recovery study was performed to assess the EDC pre-concentration protocol performance. Here an additional matrix was added that represented environmental samples more closely. Table 3.9 shows the estimated recoveries to be expected during method validation. For both filtered and unfiltered water acceptable recoveries were obtained for 8 of the 12 compounds. However, under both filtration and no filtration the SWW matrix showed recoveries never achievable. This suggests that the complex matrix might have ionization enhancing effects and therefore would first need to be quantified before accurate recoveries can be determined. The acceptable recoveries obtained from water allowed the proceeding to extensive method validation.

Finally, the pre-concentration protocol needed to be validated together with either the UPLC- or UPC^2-MS/MS instrument. While evaluating the data from the preliminary recovery study a new column for the UPC^2 arrived. Although the column chemistry differed, it was decided to do the final extensive method validation on the UPC^2-MS/MS for the following reasons: First and foremost, this is ‗n newly available instrument that will need to be reviewed. Therefore, completing the validation on this instrument will serve as publicity. Second, between the UPLC and the UPC^2, only the UPC^2 system was able successfully separate all compounds. Second to last, the UPC^2 has a reduced solvent consumption and therefore are a cheaper alternative to the UPLC. Finally, as this thesis is linked to environmental studies and the UPC^2 provides greener chemistry, this instrument will fit well into being environmentally friendly.

Extensive validation of the EDC pre-concentration, identification and quantifications was performed by investigating the conditions of sensitivity, accuracy, precision and trueness as set out in guidelines by Kruve *et al.* (43, 44). Using the sensitivity data from the preliminary studies as a guideline, calibration curves were generated to determine the LODs, LOQs, ULOQs and linearity‘s of each compound. Table 3.10 shows the relevant information. From the table it can be gathered that the sensitivity data obtained from the method validation correlate with that of preliminary findings, with only MeP showing deviations from earlier findings. Furthermore, accuracy data confers the ability of the instrument to measure all compounds qualitatively within acceptable limits (Table 3.10). In addition, each measurement was precise for all matrices tested and the deviations measured neglectable (Table 3.10). Finally the stability data in Table 3.10 show that overall all compounds, with the acception E_1, E_3 and and TCC, are stable for at least 17 hours. The latter confer minimal change to compounds and therefore infer reliability. To quantify the possible influence that environmental samples could exert on sample preparation and the final result, the matrix effects was investigated. Results are shown in **Error! Reference source not found.**. From these data we find the matrix effect for most ompounds are high and that results obtained from e.g. a WWTP could vary greatly. This data is supported by earlier finding on recovery that showed recoveries for some compounds that are never achievable. Finally, the method was evaluated for sample preparation effectiveness and the robustness of the EDC pre-concentration method. Process efficiency data in Table 3.11 show high variability between the different cartridge loads and investigators. This marks the process – from extraction to injection, as complex with room improvement and simplification. This view is strengthened by the results of Table 3.12 that show good to excellent recoveries obtainable for a number of compounds, in both water and SWW matrices. These results therefore highlight the care that should be taken in processing samples from complex matrices. Additionally, it also emphasizes that each step in the processing of samples needs to be done quantitatively.

To conclude, methods were set up on three analytical instruments and through a process of elimination the UPC^2-MS/MS was selected for the final method validation. Additionally, an EDC protocol developed, optimized and establish. Finally, the method was validated and found to be highly sensitive, accurate, precise and relatively robust, but that it lacked simplicity. From this information it is evident that the developed method can be used for environmental analysis such as the testing of samples from the JTED Ecomachine. However, it is recommended that the EDC pre-concentration protocol be simplified by doing away with steps such as the transfer between vials, as well being further optimized.

3.4 Materials and methods

3.4.1 Materials

The compounds Methyl 4-hydroxybenzoate (methylparaben, ≥99.0%), propylparaben (USP reference standard), 3,4,4'-Trichlorocarbanalide (triclocarban, 99%), Acetaminophen (paracetamol, 98.0-101.0%), Bisphenol A (analytical standard), Irgasan (triclosan, ≥97.0%), Carbamazepine (≥99.0%), 4-Nonylphenol (≥99.9%), Estrone (≥99%), 17α-Ethynyl estradiol (≥98%), β-Estradiol (≥98%) and Estriol (≥99.3%) were purchased from Sigma-Aldrich (St Louis, Missouri, USA). The compounds 4-Nonylphenol-d_4, β-Estradiol-d_5 and Carbamazepine-d_{10} used as internal standards were bought from EQ Laboratories GmbH (Augsburg, Bevaria, DE). The solvents methanol, acetonitrile, acetone and formic acid, all of analytical grade, used for SPE and chromatography were purchased from Sigma-Aldrich (St Louis, Missouri, USA), while pyridine (analytical grade) was purchased from Merck KGaA (Darmstadt, Hesse, DE). Dansyl chloride (≥99%) and N,O-Bistrifluoroacetamide (BSTFA, 1% Trimethylchlorosilane (TMCS), ≥99%) were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Supelco Supelclean ENVI-18 (6 mL, 1 g bedweight) and Supel-select HLB (6 mL, 200 mg bedweight) cartridges for SPE were bought from Sigma-Aldrich (St Louis, Missouri, USA). Meat extract (microbiological grade) and di-potassium hydrogen orthophosphate anhydrous were bought from Merck KGaA (Darmstadt, Hesse, DE), while vegetable extract no 1 (microbiological grade), D-(+)-Glucose monohydrate (≥99.0%), Magnesium sulphate heptahydrate (ACS reagent, ≥98%), Iron (II) sulphate heptahydrate (ACS reagent, ≥99.0%) and sodium chloride (BioXtra, ≥99.5% AT) were bought from Sigma-Aldrich (St Louis, Missouri, USA). Glass wool was kindly supplied by Stefan Hayward (Department of Biochemistry, University of Stellenbosch), while Whatman 41 ashless filter papers (90 mm in diameter) were bought from Sigma-Aldrich (St Louis, Missouri, USA).

3.4.2 Stock solutions

Stock solution of each compound was made by analytically weighing the appropriate amount into a 4 mL amber vial before dissolving it to a final concentration of 1000 ppm using methanol. The internal standards 4-Nonylphenol-d_4, β-Estradiol-d_5 and Carbamazepine-d_{10} were prepared in a similar manner but dissolved in acetonitrile. These solutions were further diluted with methanol or acetonitrile to 10 ppm and stored at -20 °C.

3.4.3 Instrument method development

UPLC- and UPC^2-MS/MS

Multiple reaction monitoring (MRM) were set up by directly injecting 1 µL of the pure derivatized compound at a concentration of 1 ppm into a Waters Acquity UPC^2 instrument coupled to a Waters Xevo TQ-S tandem mass spectrometer (MS/MS). Masslynx version 4.1 software was used for instrumental analysis. Derivatization was carried out according to Anari *et al.* (377). In short, the appropriate amount of each stock solution were dried with N_2 and reconstituted 1:1 with 2 mg/mL DNCl in acetone and 0.1 M $NaHCO_3$ (pH 10.5) in deionized water. The resulting solution was incubated for 10 min at 60 °C and cooled to room temperature before injection. Precursor scans were carried out between 15 and 55 V and the optimum cone voltage (CV) selected based on highest signal intensity. Using this CV, a product scan was performed between 15 and 55 V, selecting the two most prominent ions yielding the highest signal intensity at their respective collision energy (CE). The precursor CV and two product ions at their respective CE were used to construct a MRM table to monitor the different compounds. This MRM table was used for both UPC^2-MS/MS analysis and UPLC-MS/MS analysis as both are connected to the same MS. Transitions for each compound yielding the strongest signal intensity was used as quantifiers, while the rest were regarded as qualifiers.

Chromatographic separation of compounds – from a 1µL injection, using the UPC^2-MS/MS instrument was achieved on a Waters UPC^2 BEH C18 2-EP 2.1 x 100 mm, 1.7 µm pore size column – maintained at 60°C. The mobile phase consisted of solvent A (A; super critical CO_2) and solvent B (B; 1% formic acid in methanol) – supplied by a makeup pump at a rate of 0.2 ml/min. The column was equilibrated with 2% B before a linear gradient from 2 to 4.5% B, spanning 4.3 min, were applied. This gradient was followed by a 2.5 min linear gradient to 7%B and a 1 min linear gradient to 10% B, where it was kept constant for another minute. Finally, a 30% B wash step was applied for 1 min, before the column was again equilibrated for 2 min with 2% B. In contrast, separation on the Waters Acquity UPLC instrument was achieved on a Waters UPLC BEH C18 2.1 x 100 mm, 1.7 µm pore size column – maintained at 50°C. The mobile phase consisted of A (1% formic acid in ultrapure water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.35 ml/min. Prior to a 1 µL injection, the column was equilibrated with 15% B. Following injection, a 0.1 min linear gradient, from 15% to 60% B, was applied. This was followed by a 6.8 min linear gradient from 60% to 95% B, before being kept constant for 1 min. Finally, the column was equilibrated for 2 min at 15% B.

GC-MS/MS

Like UPLC- and UPC^2-MS/MS analysis, compounds and internal standards were derivatized. In short, the appropriate amount of a 10 ppm solution of each compound was dried in separate vials with N_2. Compounds and internal standards were then reconstituted in a solution consisting of two thirds pyridine and one third BSTFA, before being incubated for 60 min at 60 °C. Samples were allowed to cool to room temperature before being injected on the GC-MS/MS instrument.

Method development on GC-MS/MS was carried out on a Thermo-Fisher Scientific Trace 1300 GC, fitted with a Varian FactorFour Capilliary VF-17ms (30 m x 0.25 mm ID, DF = 0.25) column, coupled to a Thermo-Fisher Scientific TQS 8000. The retention time for each compound were determined by injecting 1 µL of each compound or internal standard in a pulsed splitless w/Surge manner under the following inlet conditions: Helium was used as carrier gas at a flow rate of 1 mL/min. The inlet temperature was set to 250 °C with a split flow of 50 mL/min after 2 min. Following injection the column was kept constant at 100 °C for 3 min, ramped to 300 °C at 10 °C/min and kept constant at 300 °C for 5 min. The MS transfer line and ion source temperature were set to 280 °C and 250 °C, respectively, with MS in full-scan mode between the m/z values of 40 and 550. AutoSRM (XCalibur, version 2.2) software was used to find the two most prominent transitions of each compound at their optimal collision energy. An SRM table was constructed from this information and used for method optimization and sensitivity determination. Finally, the transitions of each compound yielding the strongest signal intensity was used as quantifiers while the rest were used as qualifiers.

3.4.4 Instrument method optimization

UPLC- and UPC²-MS/MS

A 100 ppb mixture consisting of all 12 compounds and the three internal standards were made from each compound's 10 ppm stock solution. Aliquots of the mixture was dried and reconstituted in the same volume as the aliquot in either 1:1 (acetone:0.1 M $NaHCO_3$), 1:1 (acetonitrile:0.1 M $NaHCO_3$), 3:1 (acetonitrile:0.1 M $NaHCO_3$), 2:1:1 (acetone:acetonitrile:0.1 M $NaHCO_3$) or 1:2:1 (acetone:acetonitrile:0.1 M $NaHCO_3$), with the final concentration of DNCl equal to 1 mg/ml. These solutions were incubated at 60°C for 10 min, allowed to cool to room temperature and 1 µL injected on either the UPLC- or UPC^2-MS/MS instruments. The best solvent composition were selected for further optimization steps based on improved peak areas, signal to noise (S/N) ratios and peak shape (b/a).

One microliter of the aforementioned was injected on the UPLC-MS/MS in triplicate, the eluent flow rate changed to either 0.3 or 0.4 ml/min and triplicate injections performed again. Peak area, peak shape and S/N were re-evaluated and the best condition selected for further optimization. Similarly, column temperature (40 or 60 °C), desolvation gas flow rate (800 or 100 L/h) and desolvation gas temperature (450 or 50 °C) were evaluated. Alike, eluent flow rate (1.7 or 1.9 mL/min), desolvation gas flow rate (700 or 900 L/h) and desolvation gas temperature (450 or 550 °C) was investigated on the UPC^2-MS/MS after solvent optimization.

Results obtained from changing the different parameters were analysed with a one-way ANOVA followed by a Dunnet multiple comparisons test from the statistical package GraphPad Prism 5.00.

After the optimization of the UPC^2-MS/MS method, column failure forced an investigation into an alternative column available: a Waters Acquity UPC^2 Torus 2-PIC (3.0 x 100 mm, 1.7 µm pore size). Consequently, retention times for each compound and internal standard were determined as described in section 3.4.3 and incorporated into UPC^2-MS/MS method. No changes were made to the gradient used in section 3.4.3 or the optimal settings found in this section.

GC-MS/MS

Similar to UPLC- and UPC^2-MS/MS analysis, a 100 ppb solution of the 12 compounds and three internal standards were made and aliquots reconstituted in the same volume of either 2:1 (pyridine:BSTFA), 2:1 (Acetonitrile:BSTFA), 1:1:1 (pyridine:acetonitrile:BSTFA) or 1:4:2.5 (Pyridine:acetonitrile:BSTFA). These samples were then incubated at 60 °C for 60 min and allowed to reach room temperature before injection. In contrast to UPLC- and UPC^2-MS/MS method optimization however, two variables were investigated at the same time. First, solvent composition together with injection mode (Pulsed splitless and splitless). Second, Q1 resolution (Wide or Normal) together with emission current (50 or 75 A). Peak area, shape and S/N were evaluated in both cases and the optimal conditions selected for method sensitivity determination.

3.4.5 Preliminary sensitivity of methods developed on UPLC-, UPC^2- and GC-MS/MS

From the 10 ppm stock solutions a 250 ppb mixture was created, containing all 12 compounds and three internal standards. Consequently a dilution series was created from the 250 ppb mixture down to 1 ppt. Aliquots of the dilution series were reconstituted in the same volume of the appropriate optimized derivatizing solvent solution and incubated at and for the required temperature and time as found in the results of the previous methods section (section 3.4.4). After reaching room temperature, 1 µL of the derivatized dilution series was injected on the UPLC- and GC-MS/MS instruments. As the UPLC and UPC^2 are connected to the same instrument, sensitivity results from the UPLC were regarded as the same for the UPC^2.

A linear calibration curve was constructed by Masslynx version 4.1 (UPLC-MS/MS) or Xcalibur version 2.2 (GC-MS/MS) from the data obtained from the dilution series, with the lowest ($S/N_{quantifier}$ > 10 and $S/N_{qualifier}$ > 3) and highest data points yielding a straight line regarded as the limit of quantification (LOQ) and upper limit of quantification (ULOQ), respectively. The lowest data point yielding a $S/N_{quantifier}$ > 3, were regarded as the limit of detection. The linearity of the signal is represented as the root-mean-square value (R^2-value).

3.4.6 EDC pre-concentration from environmental matrices: method development

SPE cartridge selection

Twelve Supelclean Envi-18 and twelve Supel-select HLB cartridges were loaded with 100 mL spiked dH_2O samples according to a modified manufacturer's protocol. In short, cartridges were activated with 6 mL MeOH, equilibrated with a 6 mL aqueous solution similar to that of the sample and the sample loaded onto the cartridge. Finally compounds were eluted from the resin bed in 6 mL wash steps using different concentrations of

MeOH. Of these 24 cartridges, four Supelclean Envi-18 and four Supel-select HLB cartridges were pre-treated with MeOH and equilibrated with dH_2O acidified with 1% formic acid to a pH of 2. Consequently, 4 ng of each compound (MeP = 0.2 ng) and 5 ng of each internal standard were loaded onto the cartridge resin bed through the addition of a 100 mL sample acidified with 1% formic acid to a pH of 2. Finally, compounds were eluted from the resin bed sequentially with 5 (1% Formic acid), 10 (1% Formic acid), 20 (1% Formic acid), 50 (1% Formic acid), 60 (1% Formic acid), 80 (1% Formic acid) and 100% MeOH. The 6 mL fractions were dried with N_2 and reconstituted in 1.5 mL acetonitrile, before being transferred to separate 1.5 mL amber MS vials. Reconstituted fractions were dried with $N_{2,}$ derivatized in 200 µL 3:1 (acetonitrile:0.1 M $NaHCO_3$) and 1 µL of the solution injected on the UPLC-MS/MS instrument using the optimized instrumentation method (section 3.4.4). The remaining 8 Supelclean ENVI-18 and 8 Supel-select HLB cartridges were similarly treated. However, where 1% formic acid was used for acidification either 10 mM ammonium formate (pH 4) or 10 mM ammonium acetate (pH 6) was used.

Preliminary estimation of recoveries from Supel-select HLB cartridges under different acidification conditions

Twelve Supel-select HLB cartridges were loaded with 100 mL spiked distelled water (dH_2O) samples according to the manufacturers‘ protocol. Of these 12 cartridges, four were pre-treated with MeOH and equilibrated with dH_2O acidified with 1% formic acid to a pH of 2. This was followed by loading the sample containing 4 ng of each compound (MeP = 0.2 ng) and 5 ng of each internal standard onto the cartridge. The cartridge was washed with 60% MeOH (1% formic acid) and the compounds eluted from the resin bed with 6 mL 100% MeOH. The 6 mL fraction were dried with N_2, reconstituted in 1.5 mL acetonitrile, transferred to a 1.5 mL amber MS vial, dried with N_2 and derivatized with 200 µL 3:1 (acetonitrile:0.1 M $NaHCO_3$), before 1 µL was injected on the UPLC-MS/MS instrument using the optimized instrumentation method (section 3.4.4). The remaining 8 cartridges were treated similarly. However, where 1% formic acid was used for acidification either 10 mM ammonium formate (pH 4) or 10 mM ammonium acetate (pH 6) was used. The 1 µL injection from the differentially treated cartridges were compared to a 1 µL injection of a solvent-prepared and derivatized standard mixture, containing 20 ppb (4 ng/200 µL) of each compound and 25 ppb (5 ng/200 µL) of each internal standard.

Recoveries for each compound were calculated according to section 3.4.7 and statistical significance evaluated with a two-way no matching ANOVA followed by the Bonferroni post-test from the statistical package GraphPad Prism version 5.00.

Estimated recoveries for compounds from a water and synthetic waste water matrix

Compounds and internal standards were diluted in water and synthetic waste water (SWW) so that each 100 mL contain 4 ng of each compound (MeP = 0.2 ng) and 5 ng internal standard. The synthetic waste water matrix were adapted from van den Berg *et al.* (378) and consisted of 2182 mg/L meat extract, 218 mg/L vegetable extract, 72.7 mg/L NaCl, 182 mg/L glucose, 373 mg/L $MgSO_4•7H_2O$, 145 mg/L K_2HPO_4 and 65.9 mg/L $FeSO_4•7H_2O$.

Four 100 mL water samples (1% formic acidified to pH 2) and four SWW (1% formic acidified to pH 2) samples were pre-filtered through glass wool and Whatman 41 ashless filter paper to simulate waste water pre-treatment. Filtered samples were then loaded onto Supel-select HLB cartridges, compounds eluted from the resin bed and analysed with the optimized instrumentation method (section 3.4.4). The aqueous solution used to equilibrate the cartridge after MeOH pre-treatment were acidified with 1% formic acid to pH 2. In contrast, four more 100 mL water (1% formic acidified to pH 2) and SWW (1% formic acidified to pH 2) samples were loaded onto Supel-select HLB cartridges, compounds eluted from the resin bed and analysed with the optimized instrumentation method (section 3.4.4), without pre-filtration.

Statistical significance between filtered water, unfiltered water, filtered SWW and unfiltered SWW were determined with two-way ANOVA followed by Bonferroni post-test, using the statistical package GraphPad Prism version 5.00.

3.4.7 Comprehensive method validation

Instrumentation settings and extraction protocol

SPE column:	Supel-select HLB
Pre-conditioning:	6 mL MeOH and 6 mL 1% formic acidified dH_2O
Sample volume:	100 mL
Wash solution:	60% MeOH (1% formic acid)
Eluent:	100% MeOH
Reconstitution:	1.5 mL acetonitrile (transferred to MS vial and dried for derivatization)

Derivatization solvent: 75% acetonitrile and 25% 0.1 M $NaHCO_3$ (1 mg/ml DNCl, pH 10.5)

Derivatization volume: 200 µL

Instrument: UPC^2-MS/MS

Column: Waters UPC^2 Torus 2-PIC

Instrument setup: As set up in section 3.4.3 and optimized in section 3.4.4

Injection volume: 1 µL

Procedure

Twenty-four mixture extractions were performed by two method investigators per day per, over a three day period. Twelve of these extractions (three concentrations, 4 replicates) were from a spiked water matrix and the remaining 12 (three concentrations, 4 replicates) from a spiked pre-filtered (see section 3.4.6) SWW matrix. Compounds (from a 100 ppm stock) were diluted to the three different concentrations in the water and SWW matrices, so that each 100 mL mixture sample would theoretically contain the following amount (Table 3.13) of each compound in the derivatized sample upon injection. Each 100 mL sample also contained 5 ng of each internal standard.

Table 3.13 Low (C1), medium (C2) and high (C3) Supel-select HLB cartridge load. Final 200 µL sample concentration for injection is indicated in brackets.

Compound	Low (C1)	Medium (C2)	High (C3)
	ng (ppb)	ng (ppb)	ng (ppb)
PrP	0.02 (0.1)	0.2 (1)	2 (10)
MeP	0.002 (0.01)	0.02 (0.1)	0.2 (1)
TCS	0.02 (0.1)	0.2 (1)	2 (10)
E_1	0.02 (0.1)	0.2 (1)	2 (10)
BPA	0.05 (0.25)	0.5 (2.5)	5 (25)
EE_2	0.05 (0.25)	0.5 (2.5)	5 (25)
E_2	0.02 (0.1)	0.2 (1)	2 (10)
PCM	0.05 (0.25)	0.5 (2.5)	5 (25)
E_3	0.02 (0.1)	0.2 (1)	2 (10)
TCC	0.02 (0.1)	0.2 (1)	2 (10)

A solvent calibration series were prepared from 100 ppm stocks. First, compounds were diluted in acetonitrile, so that final concentration of each compound in the mixture would equal the highest concentration standard in the calibration range. From this solution a seven point calibration series were prepared by dilution with acetonitrile (Table 3.14). Three of the calibration points matched the theoretical concentration of the extracted samples prepared above in Table 3.13. To each of the calibration point solutions internal standards (100 ppm stocks) were added to a final concentration of 25 ppb. Two-hundred microliters of each calibration point solution was dried, reconstituted and derivatized.

Table 3.14 Solvent calibration series prepared in acetonitrile. Low (C1), medium (C2) and high (C3) Supel-select HLB cartridge load corresponds to that of Table 3.13.

Compound	C_{min} ppb	Low (C1) ppb	C_a ppb	Medium (C2) ppb	C_b ppb	High (C3) ppb	C_{max} ppb
PrP	0.05	0.1	0.5	1	5	10	20
MeP	0.005	0.01	0.05	0.1	0.5	1	10
TCS	0.05	0.1	0.5	1	5	10	20
E_1	0.05	0.1	0.5	1	5	10	20
BPA	0.125	0.25	1.25	2.5	12.5	25	50
EE_2	0.125	0.25	1.25	2.5	12.5	25	50
E_2	0.05	0.1	0.5	1	5	10	20
PCM	0.125	0.25	1.25	2.5	12.5	25	50
E_3	0.05	0.1	0.5	1	5	10	20
TCC	0.05	0.1	0.5	1	5	10	20

LOD, LOQ, ULOQ, linear range and linearity of signal

The limit of detection (LOD), limit of quantification (LOQ), upper limit of quantification (ULOQ), linear range and linearity of signal was determined, in triplicate, by injecting each derivatized solvent prepared calibration point solution. Data was analysed using Masslynx 4.1 software from the Waters Corporation. LOD, LOQ, ULOQ, linear range and linearity of signal conditions are outlined in section 3.4.5.

Precision, accuracy and trueness

Instrumental precision was determined by consecutively injecting the derivatized solvent-prepared calibration point solution, medium (C2), six times. Data analysis was performed using GraphPad Prism 5.00 and data is presented as a percentage of the relative standard deviation (% RSD). Instrumental accuracy was determined in three matrices: solvent, water and SWW.

From the water and SWW extracted samples prepared above, the medium (C2) samples of Day 1 were injected. Four separately prepared medium (C2) calibration solutions were injected in a similar way. Data analysis was performed using GraphPad Prism 5.00 and data is presented as a percentage of the relative standard deviation (% RSD).

Method trueness was assessed on four counts according to Kruve *et al.* (44): Compound stability in the final solvent, matrix effects (ionization enhancement/suppression), process efficiency and recovery. Stability was assessed by injecting a freshly prepared calibration solution, medium (C2), three times consecutively (time point zero), followed by triplicate injection of the same solution 18 hours later. Equation 1 was used to calculate each compound‘s stability.

(1)

Matrix effects were analysed by injecting each of the replicates for solvent (n= 3), water (n = 4) and SWW (n= 4) extracted low (C1), medium (C2) and high (C3) concentration of each day and each method investigator. Data analysis was performed using Masslynx 4.1 and GraphPad Prism 5.00. Mean concentrations calculated by Masslynx 4.1 were plotted against sample prepared concentrations (Table 3.13) and the gradients generated from fitted linear regression lines used in Equation 2 to calculate the matrix effect. All regression lines were test for departure from linearity with runs test.

(2)

Recovery was determined by injecting each of the replicates for water and SWW extracted low (C1), medium (C2) and high (C3) concentrations of each day and each method investigator. Matrix calibration graphs were constructed from the Masslynx 4.1 calculated mean concentrations utilizing the solvent calibration curve. Linear regression lines were plotted on these graphs and the regression line equations used calculate the original compound concentrations before extraction. Equation 3 was used to calculate recovery.

(3)

Process efficiency was determined by injecting each of the replicates for water and SWW extracted low (C1), medium (C2) and high (C3) concentrations of each day and each method investigator. The calibration solutions were used to determine the concentration of each compound in each sample and Equation 4 used to assess process efficiency.

(4)

CHAPTER 4

ELUCIDATING THE PHARMACOLOGICAL PROPERTY – DRUG POTENCY, OF FOUR ANTIMICROBIALS AND THREE HUMAN ESTROGENS ON THE YES AND E-SCREEN

4.1 Introduction and chapter objectives

In chapter 2 section 2.5.5, the endocrine disruptor (ED) effects of a wide variety of compounds were discussed as well as their possible contribution to BC carcinogenesis and progression. Chapter 3 focused on developing methods for the pre-concentration, identification and quantification of 11 of those EDCs on a newly available technology. These developed methods would prove useful in evaluating the JTED Eco-machine‘s EDC removal efficiency. This chapter however will focus on establishing techniques and gaining pharmacological information necessary to complete chapter five. The latter aims to elucidate the possible ED consequences to humans if some of those chemicals tested for in chapter two co-occur in our water resources or are applied dermally to the underarm and/or breast region. To achieve this, the pharmacological parameter for drug potency needs to be determined. The two screens discussed in chapter 2 section 2.3.2 and 2.3.3 will be used to achieve this goal. The EDCs to be tested on these screens include TCS, TCC, MeP and PrP, as they are commonly used in PCPs and can therefore find their way into the environment through the routes discussed in chapter 2 section 2.5.5. The compounds BPA, CBZ, EE_2 and 4NP will not be tested in this chapter as their inclusion in the combination studies will increase the study complexity beyond the scope of this thesis, which is to develop methods to detect EDC and to only show some of possible effects these compounds might cause to humans and animals. In addition E_1, E_2 and E_3 will also be tested on these screens as EDC-estrogen effects will also be investigated in chapter 4. Together the pharmacological information gained from these screens will be used to study drug interaction effects in chapter 4. The chapter aims and objectives are as follow:

a) Determine the drug potency concentrations of E_1, E_2, E_3, MeP, PrP, TCS, and TCC and at 25, 50 and 75% (EC_{25}, EC_{50} EC_{75}) of the maximal response.

b) Obtain repeatable results that can be used in chapter four for the mixture studies

4.2 Results

4.2.1 Yeast estrogen screen

To determine the EC_{25}, EC_{50} and EC_{75} values of compounds E_1, E_2, E_3, MeP, PrP, TCS and TCC, dose-response curves were generated from yeast exposed to a dilution series of each compound. Figure 4.1 shows a typical dose-response relationship for E_1, E_2, E_3, MeP, and PrP obtained using the YES, while Figure 4.2 and Figure 4.3 shows no dose-response properties for TCS and TCC. Results show that potency increases as follow: MeP < PrP < E_3 < E_1 < E_2 (Figure 4.1). Finally, all compounds had an efficacy close to that of E_2. **Error! Reference source not found.** shows potency data for each compound enerated from three independent YES experiments.

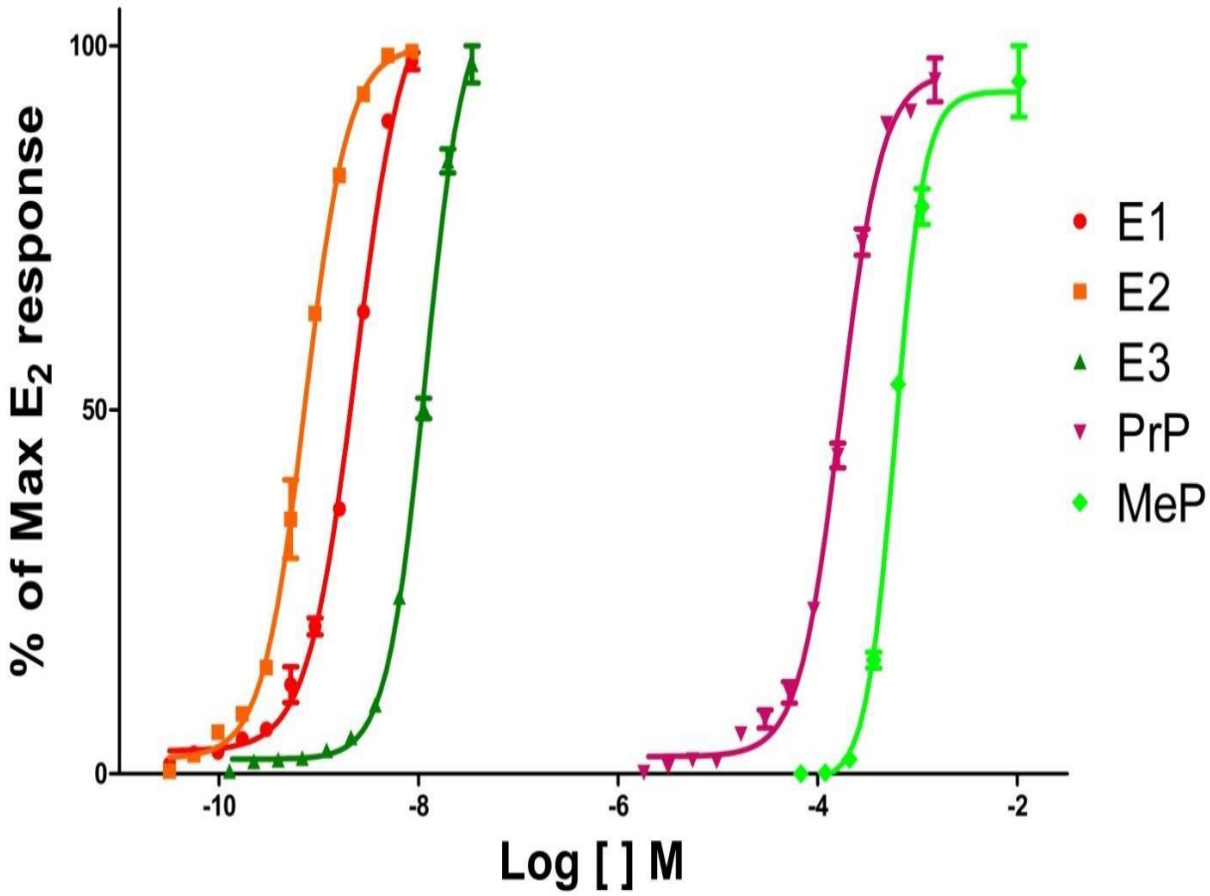

Figure 4.1 Typical dose-response curves generated from the YES for E_1, E_2, E_3, MeP and PrP. Statistical analysis and curve-fitting were carried out with the statistical package GraphPad Prism. Data points represent the mean ± SE (n =6). The log(agonist) vs. response functions were used to fit dose-responses to data.

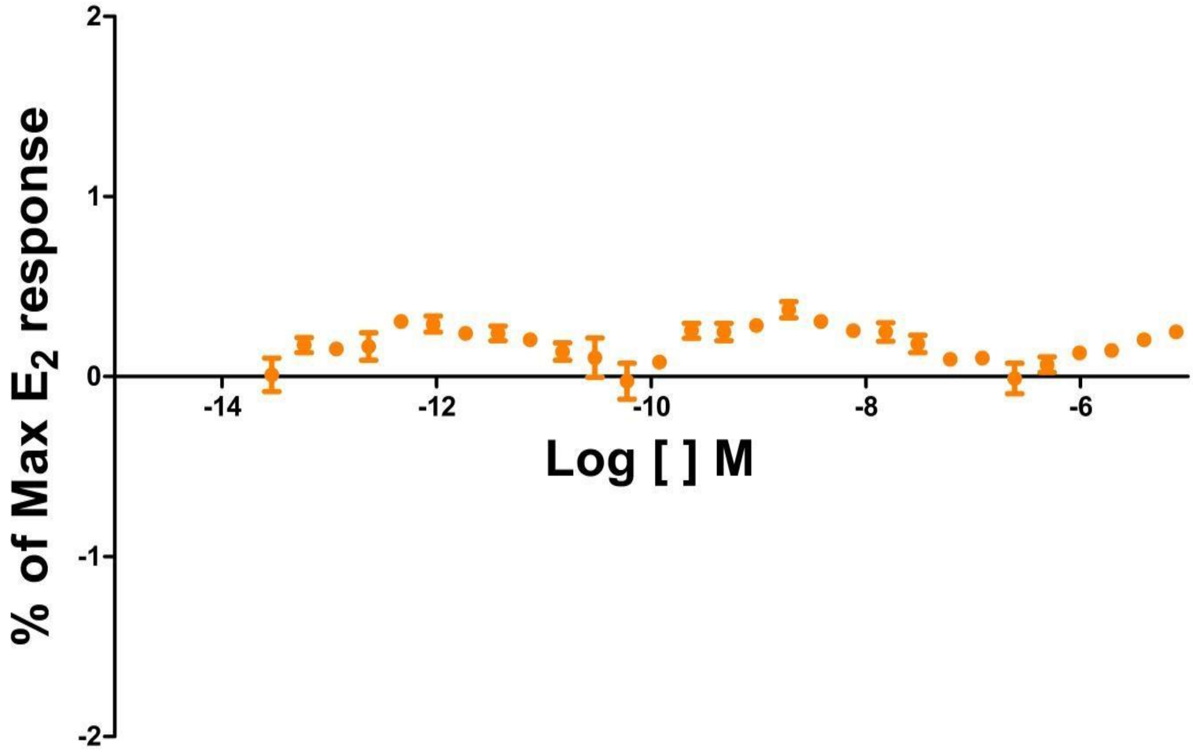

Figure 4.2 Non dose-response behaviour for TCC using the YES. Statistical analysis were carried out with GraphPad Prism and data points represent the mean ± SE (n =6).

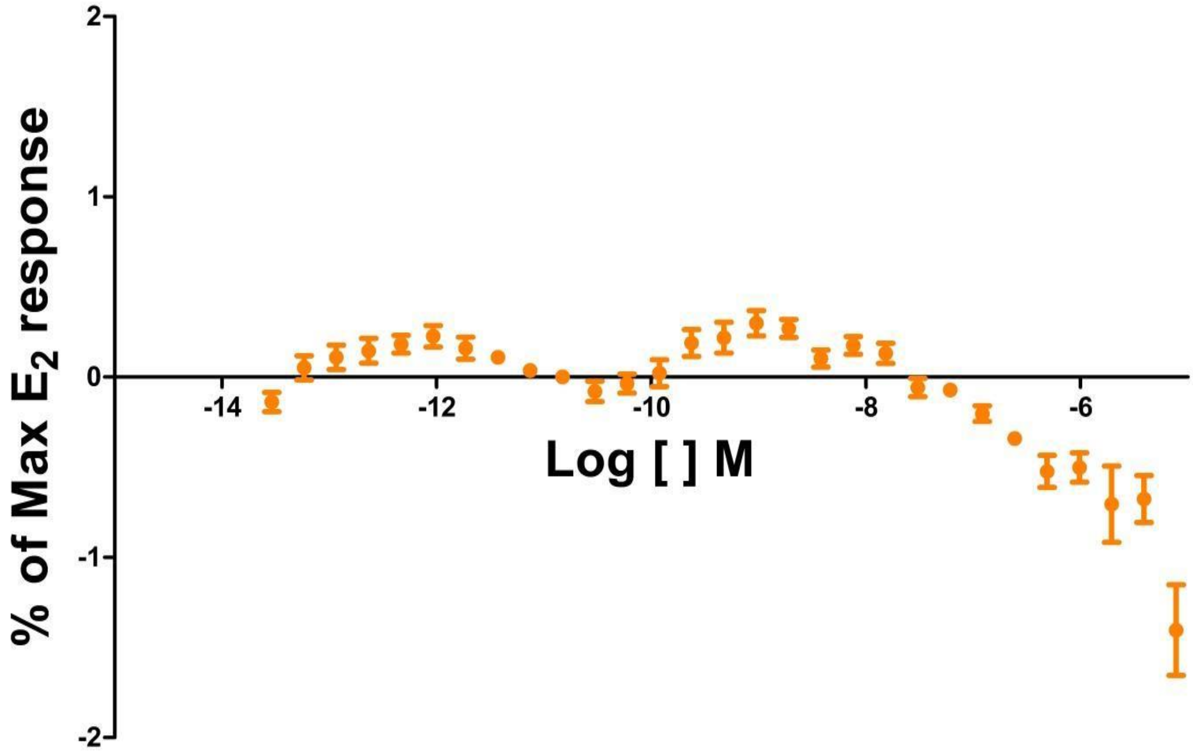

Figure 4.3 Non dose-response behaviour for TCS using the YES. Statistical analysis were carried out with GraphPad Prism and data points represent the mean ± SE (n =6). Negative values represent cytotoxic conditions.

4.2.2 E-SCREEN

Similar to the YES, MCF-7 BUS cells from the E-SCREEN were exposed to a range of compounds over a range of concentrations to determine their respective EC_{25}, EC_{50} and EC_{75} values. Figure 4.4 shows similar results for E_1, E_2, E_3, MeP, MeP and PrP to that of the YES, with the potency of compounds increasing from right to left. Table 4.2 shows the EC_{25}, EC_{50} and EC_{75} values calculated for each compound. Comparing the YES and E-SCREEN potency results, we find that all estrogens showed approximately a 200 fold higher potency when tested on the E-SCREEN, while MeP and PrP paraben potencies were approximately a 30 and 40 times higher, respectively. Finally, Figure 4.5 and Figure 4.6 indicate no dose-response behaviour for TCS and TCC, similar to results from the YES.

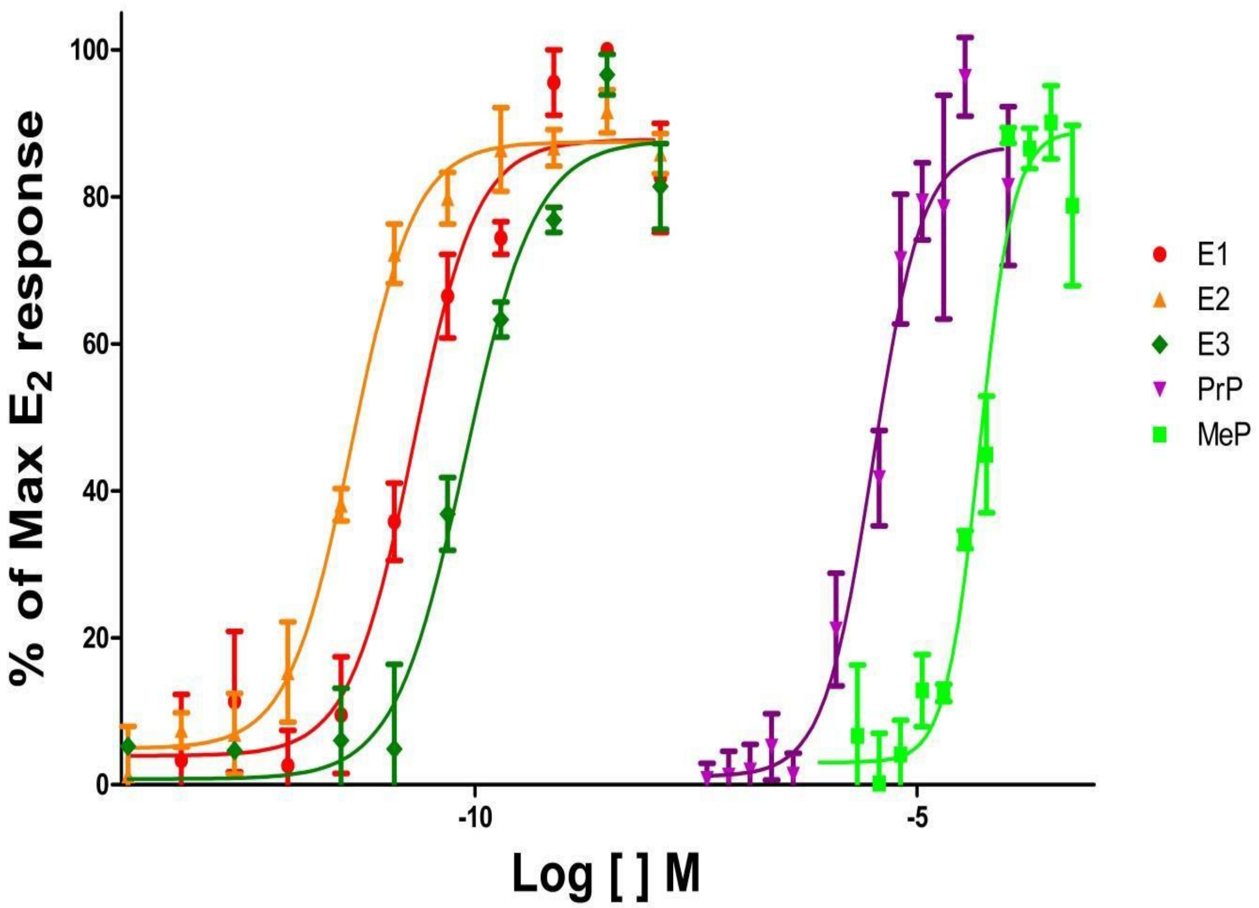

Figure 4.4 Typical dose-response curves generated from the E-screen for E_1, E_2, E_3, MeP, and PrP. Statistical analysis and curve-fitting were carried out with the statistical package GraphPad Prism. Data points represent the mean ± SE (n =6). The log(agonist) vs. response functions were used to fit dose-responses to data.

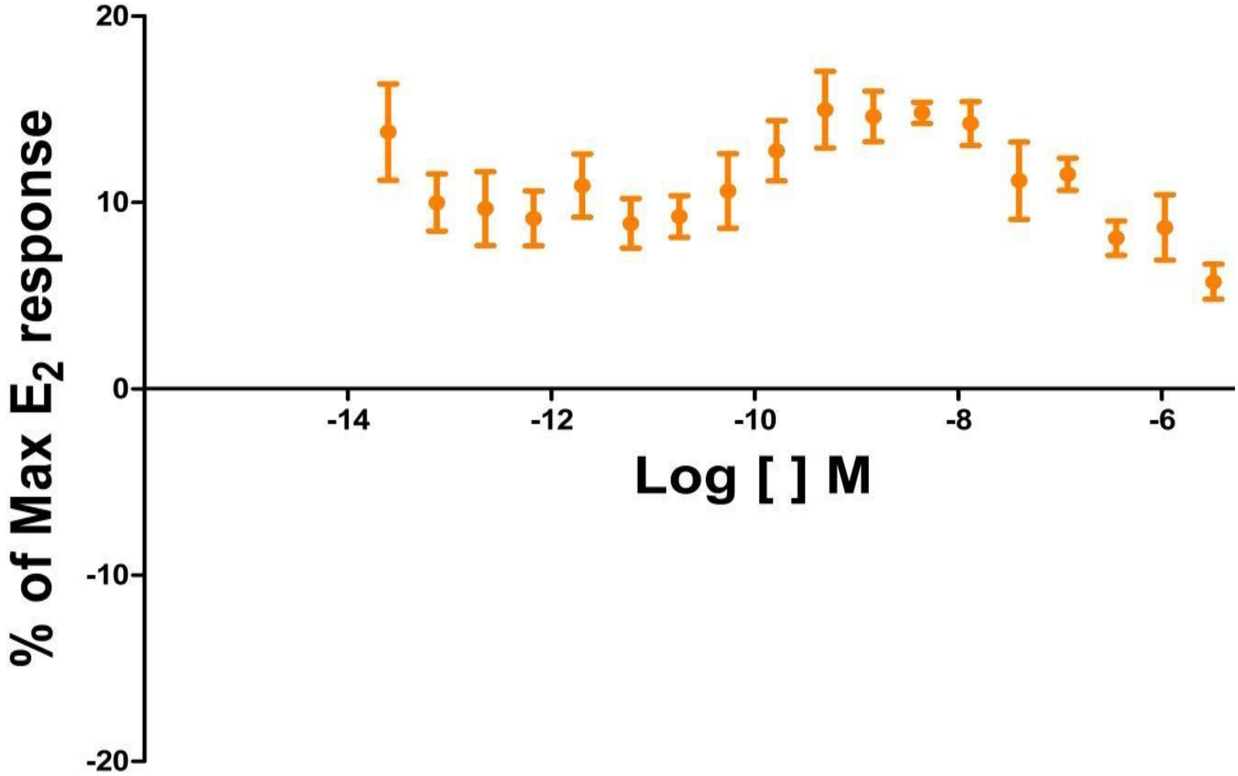

Figure 4.5 Non dose-response behaviour for TCC using the E-SCREEN. Statistical analysis were carried out with GraphPad Prism and data points represent the mean ± SE (n =3).

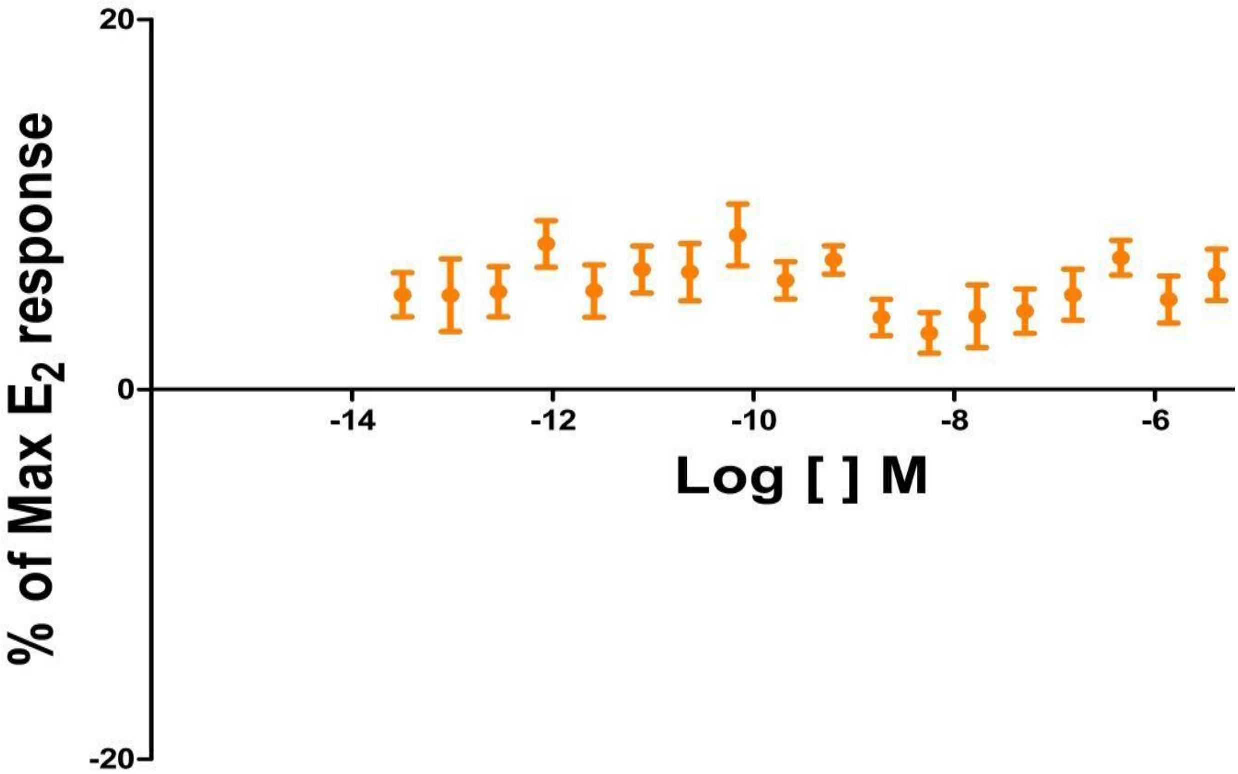

Figure 4.6 Non dose-response behaviour for TCS using the E-SCREEN. Statistical analysis were carried out with GraphPad Prism and data points represent the mean ± SE (n =3).

Table 4.1 Drug potency values for E_1, E_2, E_3, MeP, PrP, TCS and TCC with their respective curve fitting parameters. Data are the result of three independent YES experiments carried out. Statistical analysis were performed with GraphPad Prism and represent the mean ± SEM (n = 3). The average relative potency was calculated as the mean of the three relative potencies for EC_{25}, EC_{50} and EC_{75} of each compound with respect to the E_2 EC-value.

Test Compound	EC_{25} (nM)	EC_{50} (nM)	EC_{75} (nM)	Response (% of Max E_2)	Hill Coefficient	R^2 of fit	Average relative potency to E_2
E_1	1.39 ± 0.087	2.77 ± 0.212	5.49 ± 0.568	108 ± 6.59	1.62 ± 0.092	0.995 ± 0.001	0.3
E_2	0.535 ± 0.076	0.816 ± 0.090	1.33 ± 0.180	101 ± 2.34	2.42 ± 0.020	0.990 ± 0.006	1
E_3	7.25 ± 0.243	12.2 ± 0.465	20.4 ± 0.951	101 ± 0.879	2.13 ± 0.059	0.997 ± 0.002	0.1
MeP	$(2.25 \pm 0.165) \times 10^5$	$(5.28 \pm 0.279) \times 10^5$	$(6.84 \pm 0.431) \times 10^5$	80.3 ± 15.6	2.58 ± 1.07	0.972 ± 0.010	2×10^{-6}
PrP	$(7.92 \pm 1{,}44) \times 10^4$	$(1.34 \pm 0.220) \times 10^5$	$(2.27 \pm 0.356) \times 10^5$	86.7 ± 5.56	2.11 ± 0.236	0.993 ± 0.003	6×10^{-6}
TCS	Non dose-response behaviour						
TCC	Non dose-response behaviour						

Table 4.2 Drug potency values for E_1, E_2, E_3, MeP, PrP, TCS and TCC with their respective curve fitting parameters. Data are the result of three independent E-SCREEN experiments carried out. Statistical analysis were performed with GraphPad Prism and represent the mean ± SEM (n = 3). The average relative potency was calculated as the mean of the three relative potencies for EC_{25}, EC_{50} and EC_{75} of each compound with respect to the E_2 EC-value.

Test Compound	EC_{25} (pM)	EC_{50} (pM)	EC_{75} (pM)	Response (% of Max E_2)	Hill Coefficient	R^2 of fit	Average relative potency to E_2
E_1	6.28 ± 0.872	14.8 ± 2.61	34.8 ± 7.50	85.6 ± 1.58	1.31 ± 0.055	0.831 ± 0.030	0.3
E_2	1.89 ± 0.138	4.06 ± 0.053	8.81 ± 0.644	87.2 ± 0.332	1.46 ± 0.137	0.944 ± 0.009	1
E_3	31,7 ± 2.07	76.9 ± 1.58	188 ± 12.0	85.8 ± 1.77	1.25 ± 0.080	0.891 ± 0.008	0.1
MeP	$(3.07 \pm 0.249) \times 10^7$	$(5.21 \pm 0.313) \times 10^7$	$(8.97 \pm 0.888) \times 10^7$	116 ± 3.70	2.13 ± 0.214	0.959 ± 0.004	8×10^{-8}
PrP	$(1.84 \pm 0.002) \times 10^6$	$(3.25 \pm 0.002) \times 10^6$	$(5.74 \pm 0.007) \times 10^6$	114 ± 9.18	1.93 ± 0.029	0.961 ± 0.002	1×10^{-6}
TCS	Non dose-response behaviour						
TCC	Non dose-response behaviour						

4.3 Discussion

A vast number of mechanisms can be linked to ED; one of which is the binding to NRs. Modulation of certain NRs, such as ERα, have been linked to the carcinogenesis, progression and recurrence of BC. In this chapter modulation of ERα by E_1, E_2, E_3, MeP, PrP, TCS and TCC were investigated using two *in vitro* models. Results (Figure 4.1 and Figure 4.4) indicated that only E_1, E_2, E_3, MeP and PrP exhibit estrogenicity, with both models showing the potency order of compounds increasing as follow: MeP < PrP < E_3 < E_1 < E_2. Potency order for MeP and PrP are in agreement with literature showing a higher potency for PrP than MeP (344, 349, 350, 353). However, the potency order for the estrogens are somewhat of controversy – with some studies (379, 380) in agreement with results obtained in this chapter, while others (381–383) showing the order of potency to be E_1 < E_3 < E_2.

The controversy observed could be attributed to the type of model used to investigate proliferative effects. However, Lippman *et al.* (380) and Gutendorf *et al.* (382) both made use of a competitive binding assay using $[^3H]$-E_2, with minor differences in experimental protocol. Gutendorf and colleagues used 250 nM $[^3H]$-E_2, while Lippman and coworkers only used 1.46 nM $[^3H]$-E_2. The more than 170 fold difference in $[^3H]$-E_2 could explain why consensus between the assays could not be reached. Nonetheless, Gutendorf and colleagues also investigated proliferative effects of estrogens using two different luciferase-reporter-gene assays and the E-SCREEN. Both luciferase-reporter-gene assays and the E-SCREEN results correlated with their competitive binding studies. Results obtained by Escande *et al.* (383), with a luciferace-reporter-gene construct different from that of Gutendorf *et al.,* coincided with that of Gutendorf and colleagues. Data from this chapter however show results different from that attained by Gutendorf and colleagues with the E-SCREEN. The differences may be explained by four experimental differences between the protocol of Gutendorf *et al.* and the one used in this chapter. First, the cell line used by Gutendorf *et al.* was that of the original E-SCREEN protocol developed by Soto an co-workers (14), while highly responsive MCF-7 BUS cells, later developed by C. Sonnenschein and co-workers (16), were used in this chapter. The highly responsive MCF-7 BUS cells used in this chapter is highly sensitive towards estrogens or estrogen-like compounds and therefore increases the sensitivity of the experiment. Second, seeding density differed between the two protocols with only 3000 cells/mL seeded by Gutendorf *et al.* compared to 100 000 cells/ml used in this chapter from a well-established MCF-7 BUS protocol in our laboratory. Third, unlike Gutendorf *et al.*, cells were treated for five days compared to their three. Although both treatment times are in correlation with that of the original E-SCREEN assay, five days was selected instead of three to ensure proliferation is observed if treatments with EDCs yield weaker responses than that of estrogens. Finally, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay was used by Gutendorf and colleagues, while resazurin was used in this chapter to assess the metabolic activity of cells after treatment as it was readily available in the laboratory.

Error! Reference source not found. and Table 4.2 show different potencies btained for each compound using the two ERα models. EC_{50} results from **Error! Reference source not found.** for E_1 and E_2 are in accordance with the findings ($E_{1,\ EC50}$ = 3 nM and $E_{2,\ EC50}$ = 0.5 – 1.0 nM) of another yeast model used by Bovee and colleagues (381), but not for E_3 ($E_{3,\ EC50}$ = 0.6 – 1.2 nM). Furthermore, EC_{50} results in Table 4.2 for E_2 and E_3 correlate with results from a study by Gutendorf *et al.* ($E_{2,\ EC50}$ = 5 pM and $E_{3,\ EC50}$ = 70 pM) who also used the E-SCREEN. However, their data for E_1 ($E_{1,\ EC50}$ = 500 pM) differed. Other authors reporting similar results for E_1 (78 - 80 pM), E_2 (15 – 17 pM), E_3 (60 - 100 pM) using other *in vitro* cellular models, include Yang *et al.* (379), Legler *et al.* (384), van den Belt *et al.* (385) and *Gutendorf et al.* EC_{50} potencies obtained from the YES matched results (MeP_{EC50} = 0.25 mM and PrP_{EC50} = 0.15 mM) from a competitive binding assay by Blair *et al.* (344). Potencies obtained from the E-Screen was similar for PrP, but dissimilar for MeP, to results (MeP_{EC50} = 0.1 mM and PrP_{EC50} = 0.15 mM) from Charles and Darbre (350). Finally, from **Error! Reference source not found.** and Table 4.2 we ind that potencies obtained from the E-SCREEN is much higher than those of the YES. This observation can be attributed to MCF-7 BUS cells, in contrast to yeast, having multiple other nuclear pathways and targets that could lead to proliferation and therefore an increase potency value.

To conclude, this study showed that TCS and TCC have no proliferative effects as shown in Figure 4.2, Figure 4.3, Figure 4.5 and Figure 4.6. The findings from TCS are in sharp contrast to results from Gee *et al.* (321) suggesting the binding of this compound to the ER for proliferation. Much of the proliferation seen in the results of Gee *et al.* could possibly be attributed to medium not fully stripped of all estrogens or the natural state of all cells to grow and divide. Also, extended exposure of cells, normal or cancerous, to almost any compound could induce abnormalities such E_2-independent growth. This is in line with a recent publication by Sonnenschein and Soto (200). Finally, TCC results are in agreement with results from both Ahn *et al.* (329) and Chen *et al.* (330) suggesting TCC lacks agonist activity.

4.4 Materials and methods

4.4.1 Yeast estrogen screen

Materials

KH_2PO_4, $(NH_4)_2SO_4$, KOH pellets, $MgSO_4$ anhydrous, $Fe_2(SO_4)_3{\cdot}7H_2O$, $CuSO_4$ anhydrous, L-Leucine, L-Histidine, L-Arginine-HCl, L-Methionine, L-Tyrosine, L-Isoleucine, L-Lysine-HCl, L-Phenylalanine, L-Glutamic acid, L-Valine, L-Serine, L-Aspartic acid, L-Threonine, Adenine, Thiamine,-HCl, Pyridoxine, D-Pantothenic acid hemicalcium salt, Inositol, D-Biotin, D-(+)-Glucose anhydrous, Glycerol, Chlorophenol red-β-D-Galactopyranoside (CPRG) and analytical grade methanol was bought from Sigma-Aldrich (St Louis, Missouri, USA).

U-buttom 96-well microtitre plates used for the dilution series were bought from Becton Dickinson Labware (Franklin Lakes, New Jersey, USA), while the 96-well optically flatbottom microtitre plates and lids were bought from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

The test compounds, methyl 4-hydroxybenzoate (methylparaben, ≥99.0%), propylparaben (USP reference standard), Irgasan (triclosan, ≥97.0%), 3,4,4‘-trichlorocarbanalide (triclocarban, 99%), estrone (≥99%), β-estradiol (≥98%) and estriol (≥99.3%) were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

The yeast (*Saccharomyces cerevisiae* BJ1991) used in this assay was a kind gift from Professor. J. H. van Wyk (Department of Botany and Zoology, University of Stellenbosch, Stellenbosch, RSA) who obtained it from Professor J. P. Sumpter (Department of Life Sciences, Brunel University, London, UK).

Methods

Estrogen and EDC freezer stocks. All test compounds were analytically weighed and dissolved in analytical grade methanol. For ease of use, a 1000 ppm stock was prepared for each test compound, except MeP (4000 ppm), and from this a 100 ppm and 10 ppm stock created. From these stocks dilutions were made for the YES. All stocks were stored at -20 °C.

Preparation of glassware. All glassware was scrupulously cleaned with detergent and rinsed well before being cleaned twice with methanol and once with ethanol. The glassware was then sterilised for 30 min at 121 °C.

Composition of minimal medium. Minimal medium, D-(+)-Glucose (20% w/v), L-Aspartic acid (4 mg/ml), vitamin solution, L-Threonine (24 mg/mL), $CuSO_4$ (20 mM) and CPRG (10 mg/mL) was prepared according to the protocol set out by Routledge and Sumpter (9). In short: Minimal medium (pH 7.1) consisted of KH_2PO4 (13.61 g/L), $(NH_4)_2SO_4$ (1.98 g/L), KOH pellets (4.2 g/L), $MgSO_4$ (0.2 g/L), 1 ml $Fe_2(SO_4)_3$ solution (0.8 mg/ml in deionised water), L-Leucine (50 mg/L), L-Histidine (50 mg/L), Adenine (50 mg/L), L-Arginine-HCL (20 mg/L), L-Methionine (20 mg/L), L-Tyrosine (30 mg/L), L-Isoleucine (30 mg/L), L-Lysine-HCl (30 mg/L), L-Phenylalanine (25 mg/L), L-Glutamic acid (100 mg/L), L-Valine (150 mg/L), and L-Serine (375 mg/L), dissolved in deionised water. The vitamin solution comprised of thiamine (0.04 mg/mL), pyridoxine (0.04 mg/mL), pantothenic acid (0.04 mg/mL), inositol (0.2 mg/mL) and 20 ml biotin (0.02 mg/mL in deionised water) solution in deionised water. The minimal medium, D-(+)-Glucose, L-Aspartic acid and L-Threonine were sterilized at 121 °C for 30 min, while the vitamin, copper (II) sulfate and CPRG solutions were filter-sterilized with a 0.2 µm syringe filter. All solutions were stored at room temperature with the exception of the vitamin, L-Threonine and CPRG solutions that were stored at 4°C.

Yeast freezer stocks. The yeast stock obtained from J H van Wyk was added to a conical flask, containing growth medium (5 ml glucose solution, 1.25 mL L-Aspartic acid solution, 0.5 mL vitamin solution, 0.4 mL L-Threonine and 125 µL copper (II) sulfate solution and 45 mL minimal medium), prior to incubation at 26 °C for 24 hours on an orbital shaker. After 24 hours, 1 mL of the overnight culture was added to two conical flasks, with fresh growth medium, and incubated for another 24 hours at 26 °C on the orbital shaker. On day 3, the overnight cultures were transferred to 50 mL centrifuge tubes and the cultures spun down at 4 °C for 10 min at 2000 x g using an Avanti JE centrifuge. The supernatant was decanted and each pellet resuspended in 5 mL minimal medium with 15% glycerol. Aliquots of 500 µL (10X concentrated) were stored at -20 °C for a maximum of 4 months before new stocks were made from the existing ones and the old discarded.

YES protocol. On day one, a 125 µL of the 10X concentrated yeast stock were incubated in growth medium for 24 hours on an orbital shaker at 26 °C. After 24 hours the yeast were passaged by incubating 1 mL of the overnight culture at 26 °C on an orbital shaker in fresh growth medium. On day three, test chemicals were serially diluted in a 96-well U-bottom microtitre plate with methanol as needed. Ten microliter aliquots were then transferred to sterile 96-well optically flat bottom microtitre plates and left to evaporate to dryness (Coated wells). Ten microliter aliquots of methanol were transferred to blank wells, as negative control, and evaporated to dryness. β-Estradiol were used as positive control in all assays and are treated in a similar manner as test compounds before being evaporated. The assay medium (0.5 mL CPRG and 2 mL yeast per 50 mL fresh growth medium) was seeded according to the number of 96-well plates needed (see Table 4.3). To the coated wells, 200 µL of the seeded assay medium was added, prior to sealing the 96-well plates with autoclave tape. Finally, plates were shaken for 30 min on an orbital shaker before being incubated at 30 °C until half the wells of the positive control changed colour (2-5 days).

Table 4.3 Composition of assay medium for different plate sizes

Assay constituents	½ Plate	1 Plate	3 Plates	4.5 Plates
Minimal Medium (mL)	9	18	54	81
Glucose (mL)	1	2	6	9
$CuSO_4$ (µL)	25	50	150	225
L-Aspartic acid (mL)	0.25	0.5	1.5	2.25
Vitamin solution (µL)	100	200	600	900
L-Threonine (µL)	80	160	480	720
CPRG (µL)	100	200	600	900
Yeast (mL)	0.4	0.8	2.4	3.6

Spectrophotometric readings. To correct for turbidity absorbance (abs.) readings were taken at 570 and 620 nm using a BioRad microtitre plate reader (BioRad Laboratories Ltd., Hemel Hempstead UK) and Equation 5 applied to the data. Consequently, the median of all blanks were subtracted from wells containing test compounds and the data expressed as a percentage of the maximum E_2 observed in the experiment.

$$(\quad)\ [\ (\quad)\ \quad] \qquad (5)$$

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Limit of detection and limit of quantification. Data used in the fitting of dose-response curves were considered quantifiable for all data points where the absorbance measurement of the sample was greater than the negative control plus three times the standard deviation.

Statistical analysis. Statistical analysis was carried out with the statistical package GraphPad Prism 5.00. The same statistical package was also used to fit data to dose-response curves. Here, the functions log(agonist) vs. response – variable slope and log(agonist) vs. response – find ECanything, were used to find the potencies (EC_{25}, EC_{50} and EC_{75}) for each compound.

4.4.2 E-SCREEN

Materials

Dulbecco's Modified Eagles Medium (DMEM, low glucose and L-glutamine, without sodium bicarbonate and phenol red), DMEM (high glucose, glutamine and phenol red, without sodium bicarbonate), Sodium pyruvate (BioReagent, suitable for cell culture, ≥ 99%), D-(+)-Glucose (Hybri-Max, ≥ 99.5%), bovine calf serum (iron supplemented) and resazurin sodium salt were bought from Sigma-Aldrich (St Louis, Missouri, USA). Penicillin Streptomycin and TrypLE^{TM} express enzyme and Trypan blue stain (0.4%) were bought from Life Technologies (Grand Island, NY, USA), while sodium hydrogen carbonate was bought from Merck KGaA (Darmstadt, GE).

Both the T75 flasks used for the maintenance of cells and experimental 96-well sterile plates (F-bottom, with lid) were bought from Sigma-Aldrich (St Louis, Missouri, USA).

The test compounds, methyl 4-hydroxybenzoate (methylparaben, ≥ 99.0%), propylparaben (USP reference standard), Irgasan (triclosan, ≥97.0%), 3,4,4'-trichlorocarbanalide (triclocarban, 99%), estrone (≥99%), β-estradiol (≥98%) and estriol (≥99.3%) were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

The MCF-7 BUS (Passage number 181) cell line used in the E-SCREEN was a kind gift from Dr D. Africander (Department of Biochemistry, University of Stellenbosch, Stellenbosch, RSA), who obtained it from Professor A. M. Soto (Department of Integrative Physiology and Pathobiology, Tufts University, Boston, USA).

Methods

Estrogen and EDC freezer stocks. All test compounds were analytically weighed and dissolved in analytical grade ethanol. For ease of use, a stock concentration of 1 mM was prepared of all test compounds, except TCC for which a 30 mM stock was made. From these stocks dilutions were made with ethanol that was a 100-1000 fold higher than the concentration used in experiments. The 100-1000 fold higher stocks were then diluted in media before being plated in wells. All ethanol stocks were stored at -20°C.

Preparation of glassware. All glassware was thoroughly cleaned with 2% Contrad and rinsed well before being cleaned twice with methanol and once with ethanol. The glassware was then sterilised for 30 min at 121°C.

Composition of Growth and experimental medium. Growth medium (GM) consisted of Dulbecco's Modified Eagles Medium (DMEM; High glucose with phenol red) supplemented with 1.5 g/L $NaHCO_3$, 0.11 g/L sodium pyruvate, 5% Heat inactivated (HI) Fetal Calf Serum (FCS) and 10% Penicillin-Streptomycin (PenStrep). DMEM supplemented with 1.5 g/L $NaHCO_3$, 0.11 g/L sodium pyruvate was filter-sterilized with a 0.22 µm filter and stored at 4 °C for extended periods. Before use, the remaining supplements were added and the medium stored at 4°C for no longer than 6 months. Experimental medium consisted of Dulbecco's Modified Eagles Medium (DMEM; Low glucose without phenol red) supplemented with 1.5 g/L $NaHCO_3$, 3.5 g/L Glucose and 5% HI Charcoal-Dextran-Coated (DCC) stripped FCS. DMEM supplemented with 1.5 g/L $NaHCO_3$ and 3.5 g/L glucose was filter-sterilized with a 0.22 µm filter and stored at 4 °C under dark conditions for extended periods. Before use, the remaining supplement was added and the medium stored at 4 °C under dark conditions for no longer than 6 months.

MCF-7 BUS freezer stocks. MCF-7 BUS cells were maintained in GM until cell semi-confluent for stock solutions to be created. Following medium removal, cells were detached from the surface of flasks using TrypLE™ express enzyme and diluted in 10 mL GM. Consequently cells were pelleted at 2000 rpm over 5 min and resuspended in the 10 mL GM. Cells were counted by staining with Trypan blue before counting on a Countess automatic cell counter (Invitrogen). Freezer stocks were made by diluting cells to 1 x 10^6 cells/mL with GM containing 7% Dimethyl sulfoxide (DMSO). Long term storage of cells was carried out in liquid nitrogen, while stocks for regular use were stored at -80 °C until use.

E-SCREEN protocol. MCF-7 BUS cells were maintained in GM before being plated in 96-well plates at a seeding density of 100 000 cells/ml. Semi-confluent cells were detached from flasks with TrypLE™ Express enzyme and resuspended in 10 mL GM, before being pelleted. Pelleted cells were resuspended in 10 mL fresh GM, counted as mentioned before and diluted with GM to a final concentration of 100 000 cells/mL. Each well of the 96-well plate received a 100 µL seeded GM (10 000 cells) Plates were incubated for 24 hours at 37 °C and 5% CO_2 before the GM was replaced with 200 µL EM and incubated under the same conditions. After 24 hours the medium was removed and replaced with 100 µL fresh EM, containing the compound of interest, an ethanol control and E_2 positive control. Treated plates were incubated for 120 hours before 20 µL of sterile resazurin solution (0.15 mg/mL in phosphate buffered saline, pH 7.1) was added per well. Plates were incubated for 3-4 hours at 37 °C and 5% CO_2 before spectrophotometric readings were taken with a Tecan Spark 10M (Tecan Trading AG, Switzerland EU).

Spectrophotometric readings. After the final incubation step fluorescence readings were taken 570 and 600 nm and blanks subtracted from treated wells. Data was expressed as a percentage of the maximum E_2 observed in the experiment.

Limit of detection and limit of quantification. Data used in the fitting of dose-response curves were considered quantifiable for all data points where the fluorescence measurement of the sample was greater than the negative control plus three times the standard deviation.

Statistical analysis. Statistical analysis was carried out with the statistical package GraphPad Prism 5.00. The same statistical package was also used to fit data to dose-response curves. Here, the functions log(agonist) vs. response – variable slope and log(agonist) vs. response – find ECanything, were used to find the potencies (EC_{25}, EC_{50} and EC_{75}) for each compound.

CHAPTER 5

INVESTIGATING THE COMBINATORY EFFECTS OF PCPs AND THEIR POSSIBLE CONTRIBUTION TO BREAST CANCER

5.1 Introduction and chapter objectives

In chapter 2 section 2.5.5, the ED effects of a number of EDCs, their modes of action and contribution to BC were discussed. Chapter 4 was dedicated to investigating the ERα dose-response properties of MeP, PrP, TCS and TCC – a subset of the discussed EDCs as well as E_1, E_2 and E_3, using two ERα models. Results showed that MeP and PrP both had dose-response properties similar to that of E_1, E_2 and E_3, but that both MeP and PrP had lower potencies than the estrogens. It is well documented that MeP, PrP, TCS and TCC have been found in both normal and cancerous breast tissue as well as breast milk (315, 340–342, 386–389). The presence of estrogens in the breast, or for that matter EDCs with estrogenic potential, can therefore play a deciding role in the progression of BC. It has also been shown that certain EDC such as MeP can actually inhibit the function of anti-estrogens prescribed to BC patients (357), emphasizing the need to do away with preservatives exhibiting ED effects. Furthermore, a study has also showed how low doses of a combination of EDC can sometimes have far more devastating effects than the compounds in isolation (350). An expert panel later suggested that a mixture of EDCs should be considered rather than compounds in isolation (68). These views and findings then also correlate with the way that EDCs are found in waste water such as that draining into the Berg River.

This chapter aims to do just that: investigate the effects of a mixture of EDCs, with the compounds under investigation being MeP, PrP, TCS and TCC. The goal of this study was to investigate the combinatory effects mediated through ERα, by looking specifically at how each EDC can modulate ERα in the presence of estrogens as well as the presence of other EDCs. The aims and objectives of this chapter are as follow:

a) Using the YES:
   - Determine the ERα modulatory effects of combinations between MeP, PrP, TCS or TCC with the estrogens.
   - Determine the ERα modulatory effects of combinations between MeP and PrP, as well as TCS and TCC.
   - Determine the ERα modulatory effects of combinations between MeP or PrP with TCS or TCC.

5.2 Results

To investigate the combinatory effects between MeP, PrP, TCS, TCC, E_1, E_2 and E_3 yeast expressing ERα were treated with binary mixtures of the different compounds. With the exception of combination with TCS and TCC, all compounds were treated so that the combination would theoretically yield additive effects equal to 100% E_2. Concentrations previously investigated in literature (329) were used for combination studies with TCS and TCC. Figure 5.1, Figure 5.2 and Figure 5.3 show the combinatory effects between the parabens and estrogens, Figure 5.4, Figure 5.5 and Figure 5.6 the effects between TCS, TCC and the estrogens and Figure 5.7 and Figure 5.8 the effects between TCS, TCC and the parabens, while Figure 5.9 show the effects between the parabens or TCS and TCC.

MeP and PrP in combination differentially affected the modulation of ERα. First, MeP at its EC_{75} potency, as well as in combination with the EC_{25} value of E_1 showed no stimulatory effect and instead seemed to indicate cytotoxicity. In contrast, PrP on its own stimulated the modulation of ERα, while in combination with E_1 showed antagonistic properties. All remaining combination between MeP and E_1 showed ERα antagonistic activity, where the combined effect yielded a lower response than that of the individual compounds. However, when combining PrP at doses higher than its EC_{25} potency with doses for E_1 lower than its EC_{75} potency, additivity was achieved. Similar results were obtained when combining MeP or PrP with E_2, with only one difference: additivity was observed when combining MeP and E_2 at their respective EC_{75} and EC_{25} potencies. Different results were observed for combination between MeP or PrP and E_3.In isolation, MeP at its EC_{75} or E_3 at its EC_{25} potency showed no response and neither did the combination. Furthermore, the stimulatory effect observed for E_3 was low at all three concentrations. Combining MeP with E_3, both at EC_{50}, results suggest antagonism while synergism was observed for MeP at its EC_{25} potency and E_3 at its EC_{75} potency. Finally, combinations with PrP and E3 showed additivity (PrP_{EC50} + $E_{3,\ EC50}$) as well as synergism (PrP_{EC25} + $E_{3,\ EC75}$).

The combinatory effect between TCS or TCC and the estrogens were subsequently investigated. Combinations with TCS showed antagonistic properties for all but one combination: 0.1 µM TCS in combination with E_1 at its EC_{75} potency, which showed and additive response. In contrast, all combinations with TCC and E_1 indicated a synergistic behaviour. TCS combinations with E_2 resulted in responses showing either no response or antagonism. Like the combinations between E_1 and TCC, all but one ($TCC_{0.1\ \mu M}$ + $E_{2,\ EC75,}$ additivity) combination between TCC and E_2 showed synergistic effects. Interestingly, although the overall response was low, TCS_{EC75} combined with $E_{3,\ EC25}$ showed a synergistic response. This is in contrast to results from TCS combination with E_1 or $E_{2,}$ as well as the combination between TCS at its EC_{25} potency and E_3 at its EC_{75} potency. Finally, TCC combined with E_3 resulted in responses similar to that obtained by combination between TCC and E_2.

Lastly, combinations between the different EDCs were tested. All combinations between MeP and TCS showed antagonistic behaviours, while combinations between MeP and TCC showed antagonistic (MeP_{EC50} + $TCC_{1.0\ \mu M}$) and synergistic (MeP_{EC25} + TCC_{10}) behaviour. Similarly to combinations with MeP, combinations with PrP and TCS showed mostly antagonistic properties. In contrast, PrP combined with TCC showed both antagonistic (PrP_{EC75} + $TCC_{0.1\ \mu M}$ and PrP_{EC50} + $TCC_{1.0\ \mu M}$) and synergistic ($TCC_{10\ \mu M}$ + PrP_{EC75}) properties. To conclude, all combinations between MeP and PrP showed antagonism, while most combinations between TCS and TCC showed antagonistic properties. The only exception was the combination between 0.1 µM TCS and 10 µM TCC that showed synergism.

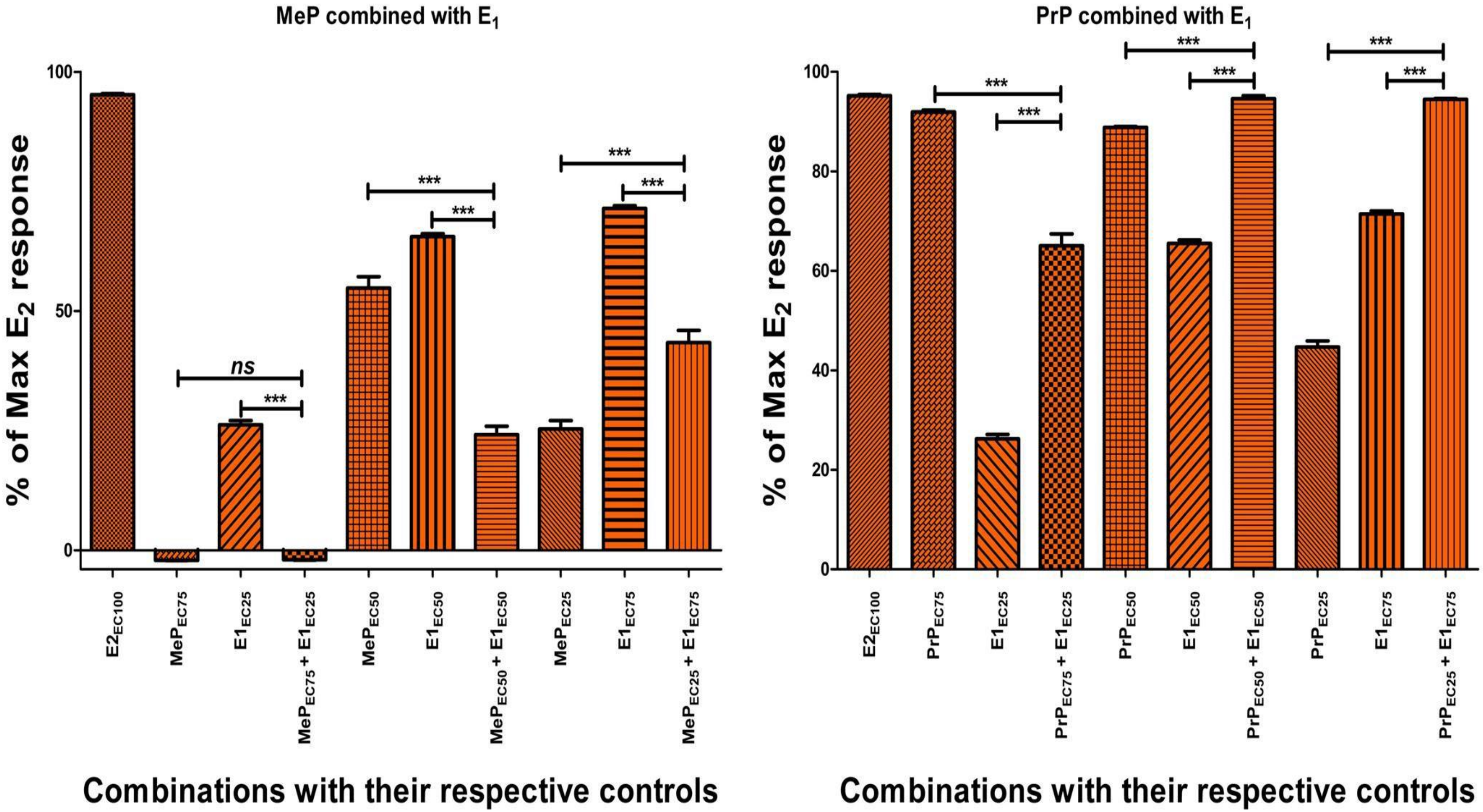

Figure 5.1 ERα modulatory effects observed when combining MeP or PrP with E_1 using the YES. Data shown are represented as the mean ± SE (n = 10), Statistical analysis was performed with one-way ANOVA followed by a Dunnett post-test. Significance are denoted by *, ** and *, corresponding to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Negative values represent cytotoxic conditions. The cytotoxic effects observed for MeP_{EC75} could possibly be attributed to experimental error whereby the concentration was higher than the EC_{75} value, resulting in cytotoxic effects.**

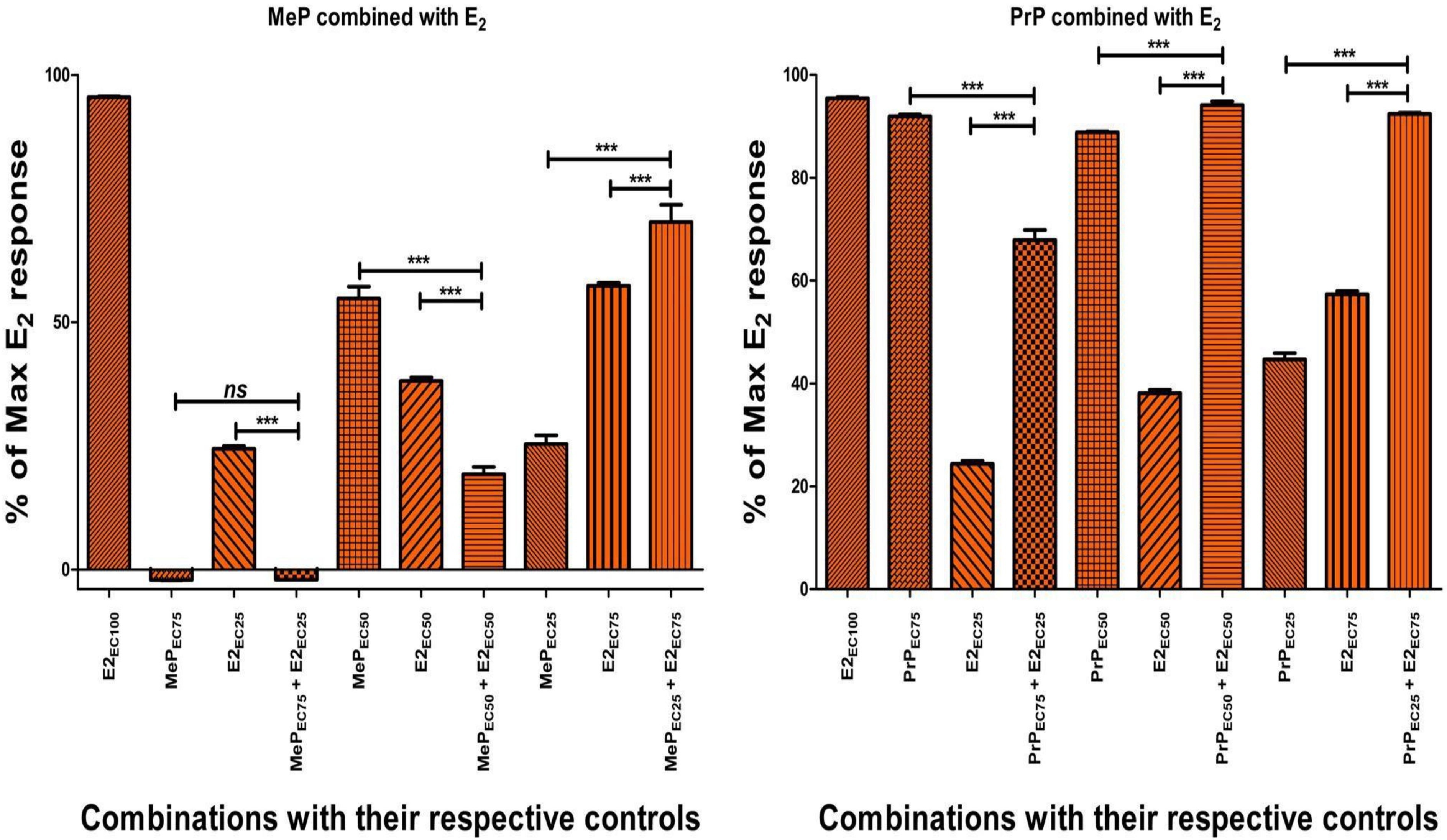

Figure 5.2 ERα modulatory effects observed when combining MeP or PrP with E_2 using the YES. Data shown are represented as the mean ± SE (n = 10), Statistical analysis was performed with one-way ANOVA followed by a Dunnett post-test. Significance are denoted by *, ** and *, corresponding to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Negative values represent cytotoxic conditions. The cytotoxic effects observed for MeP_{EC75} could possibly be attributed to experimental error whereby the concentration was higher than the EC_{75} value, resulting in cytotoxic effects.**

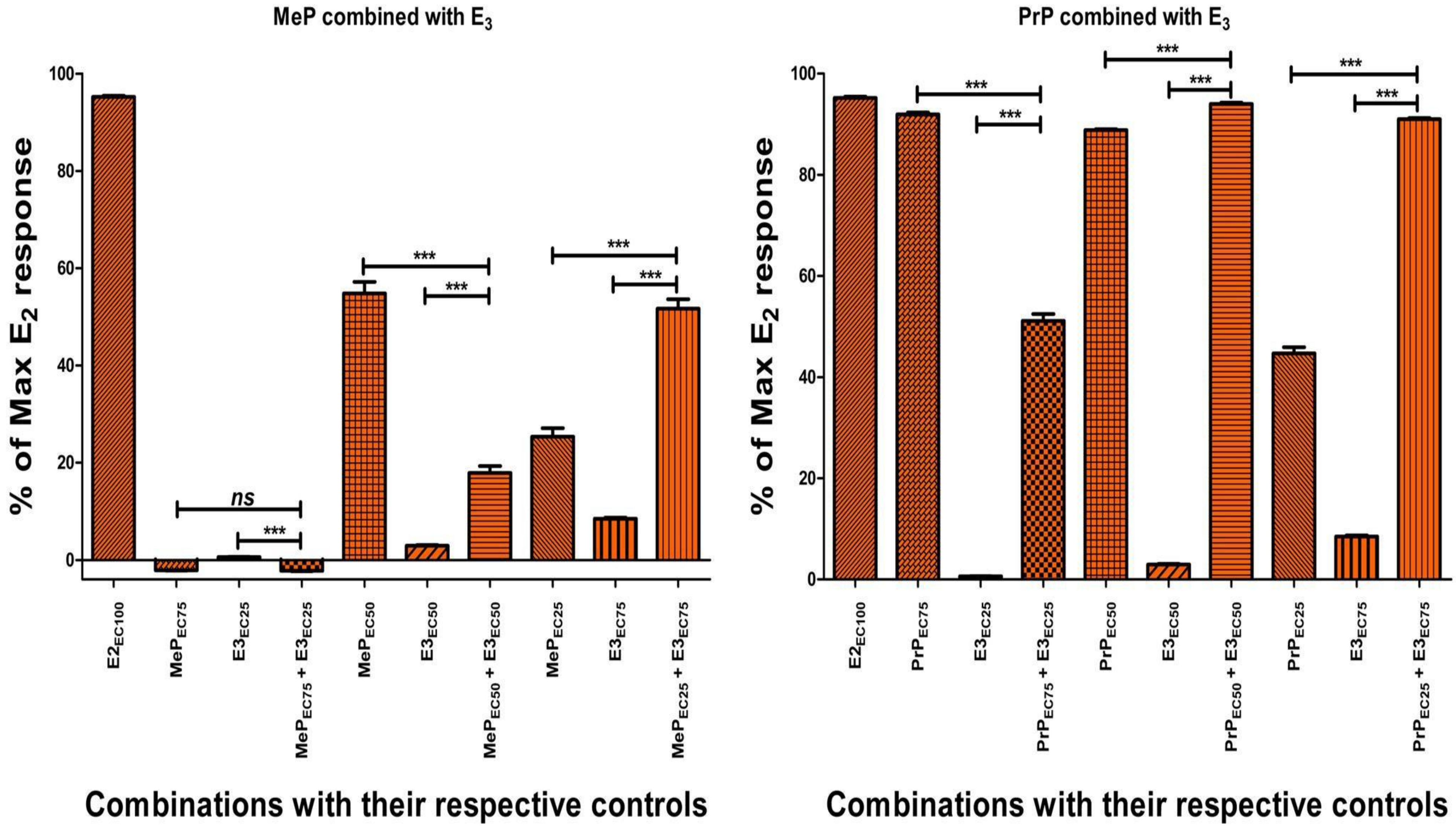

Figure 5.3 ERα modulatory effects observed when combining MeP or PrP with E_3 using the YES. Data shown are represented as the mean ± SE (n = 10), Statistical analysis was performed with one-way ANOVA followed by a Dunnett post-test. Significance are denoted by *, ** and *, corresponding to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Negative values represent cytotoxic conditions. The low responses observed for $E_{3,EC25}$, $E_{3,EC50}$ and $E_{3,EC75}$ could possibly be attributed to experimental error whereby the concentration was lower than the values calculated for in chapter 4.**

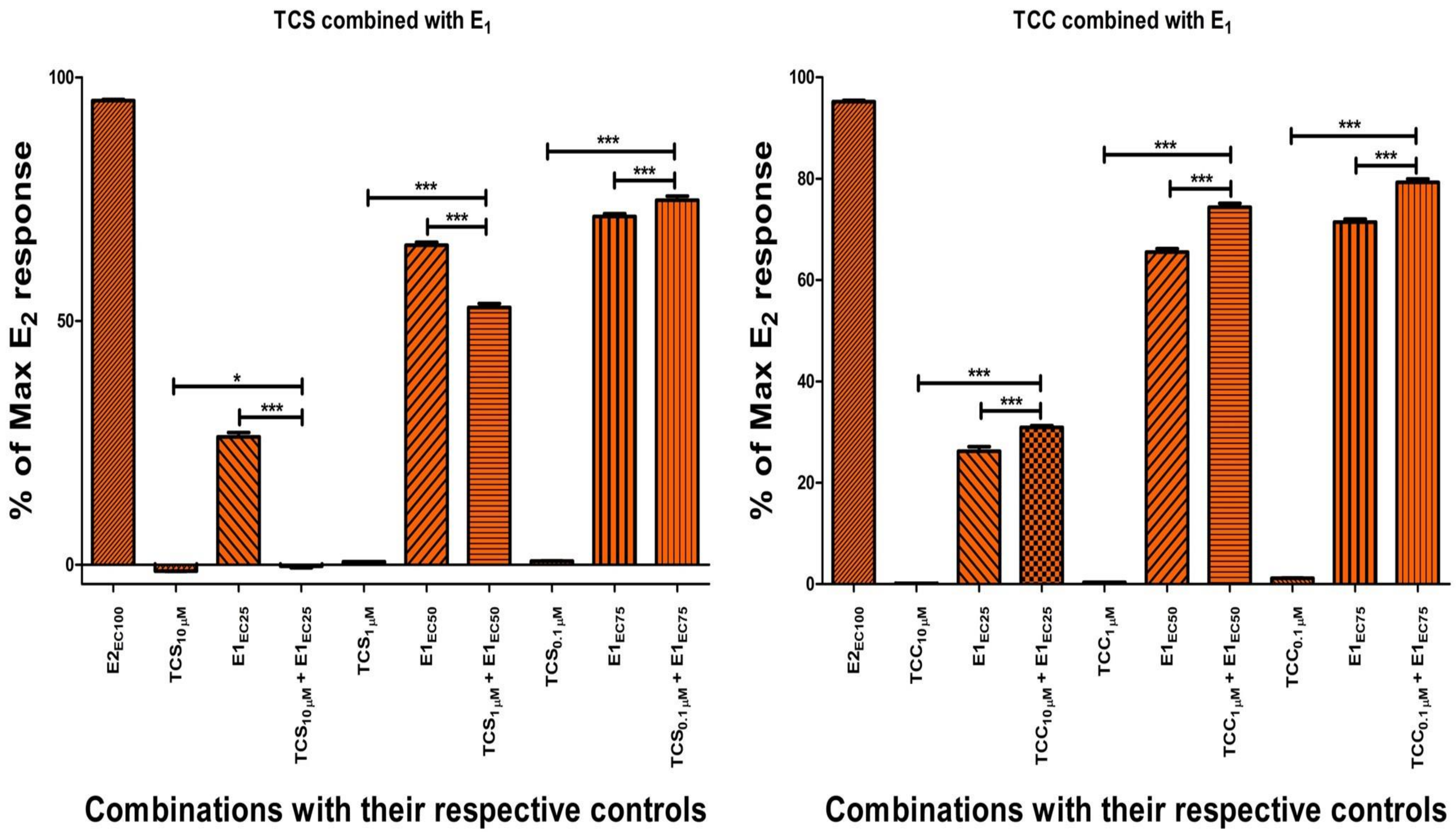

Figure 5.4 ERα modulatory effects observed when combining TCS or TCC with E_1 using the YES. Data shown are represented as the mean ± SE (n = 10), Statistical analysis was performed with one-way ANOVA followed by a Dunnett post-test. Significance are denoted by *, ** and *, corresponding to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Negative values represent cytotoxic conditions.**

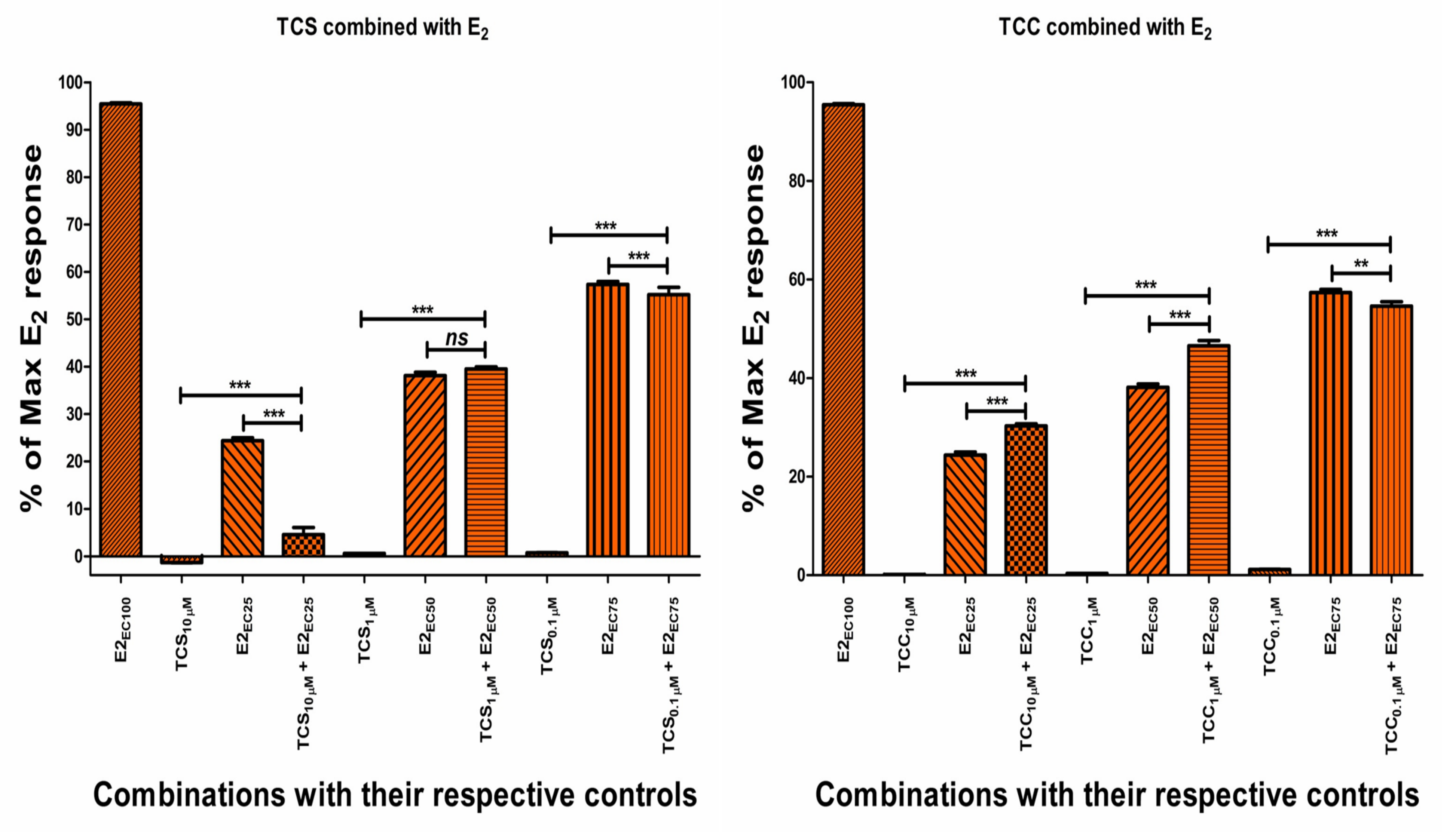

Figure 5.5 ERα modulatory effects observed when combining TCS or TCC with E_2 using the YES. Data shown are represented as the mean ± SE (n = 10), Statistical analysis was performed with one-way ANOVA followed by a Dunnett post-test. Significance are denoted by *, ** and *, corresponding to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Negative values represent cytotoxic conditions.**

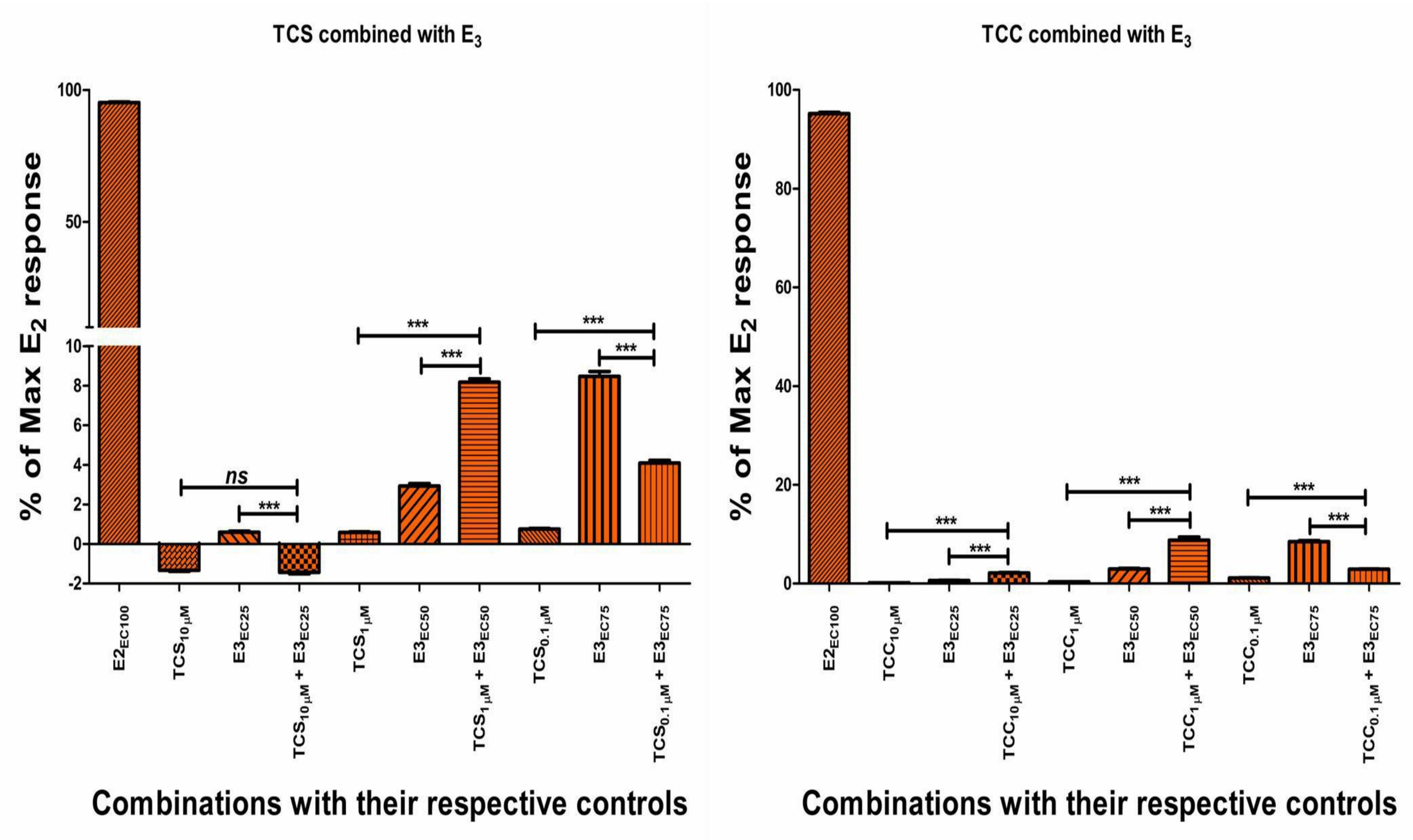

Figure 5.6 ERα modulatory effects observed when combining TCS or TCC with E_3 using the YES. Data shown are represented as the mean ± SE (n = 10), Statistical analysis was performed with one-way ANOVA followed by a Dunnett post-test. Significance are denoted by *, ** and *, corresponding to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Negative values represent cytotoxic conditions.**

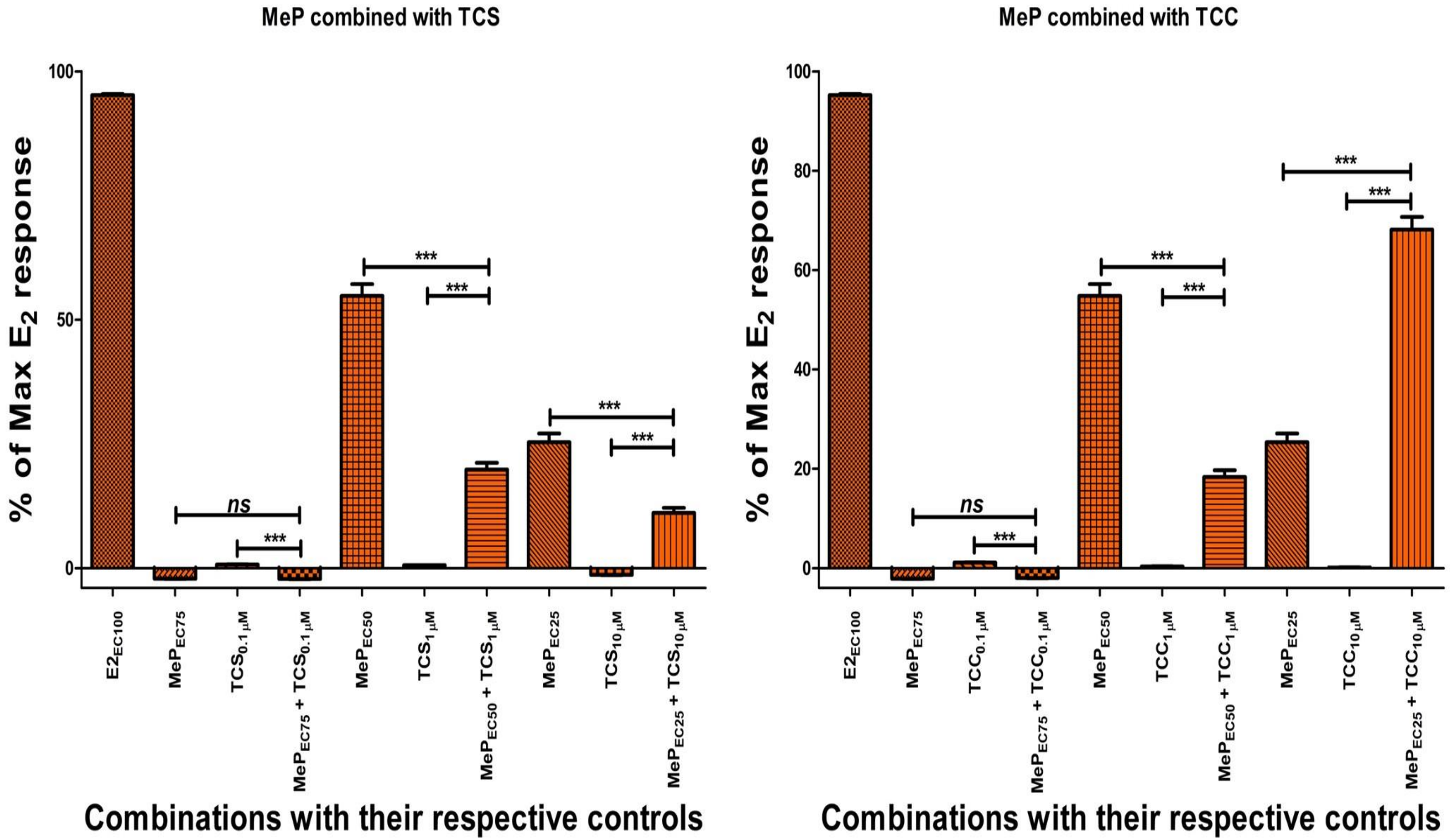

Figure 5.7 ERα modulatory effects observed when combining TCS or TCC with MeP using the YES. Data shown are represented as the mean ± SE (n = 10), Statistical analysis was performed with one-way ANOVA followed by a Dunnett post-test. Significance are denoted by *, ** and *, corresponding to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Negative values represent cytotoxic conditions.**

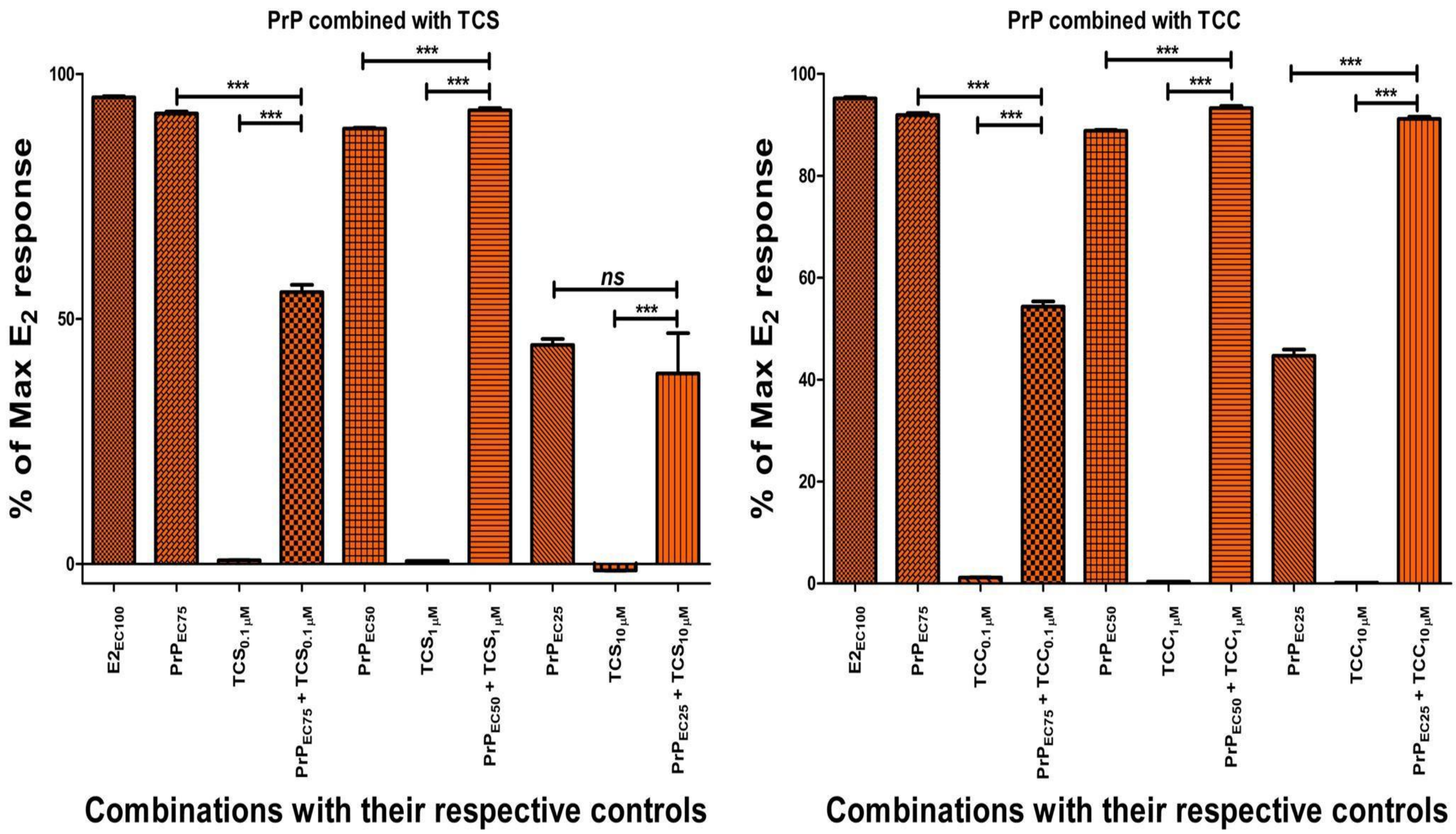

Figure 5.8 ERα modulatory effects observed when combining TCS or TCC with PrP using the YES. Data shown are represented as the mean ± SE (n = 10), Statistical analysis was performed with one-way ANOVA followed by a Dunnett post-test. Significance are denoted by *, ** and *, corresponding to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Negative values represent cytotoxic conditions.**

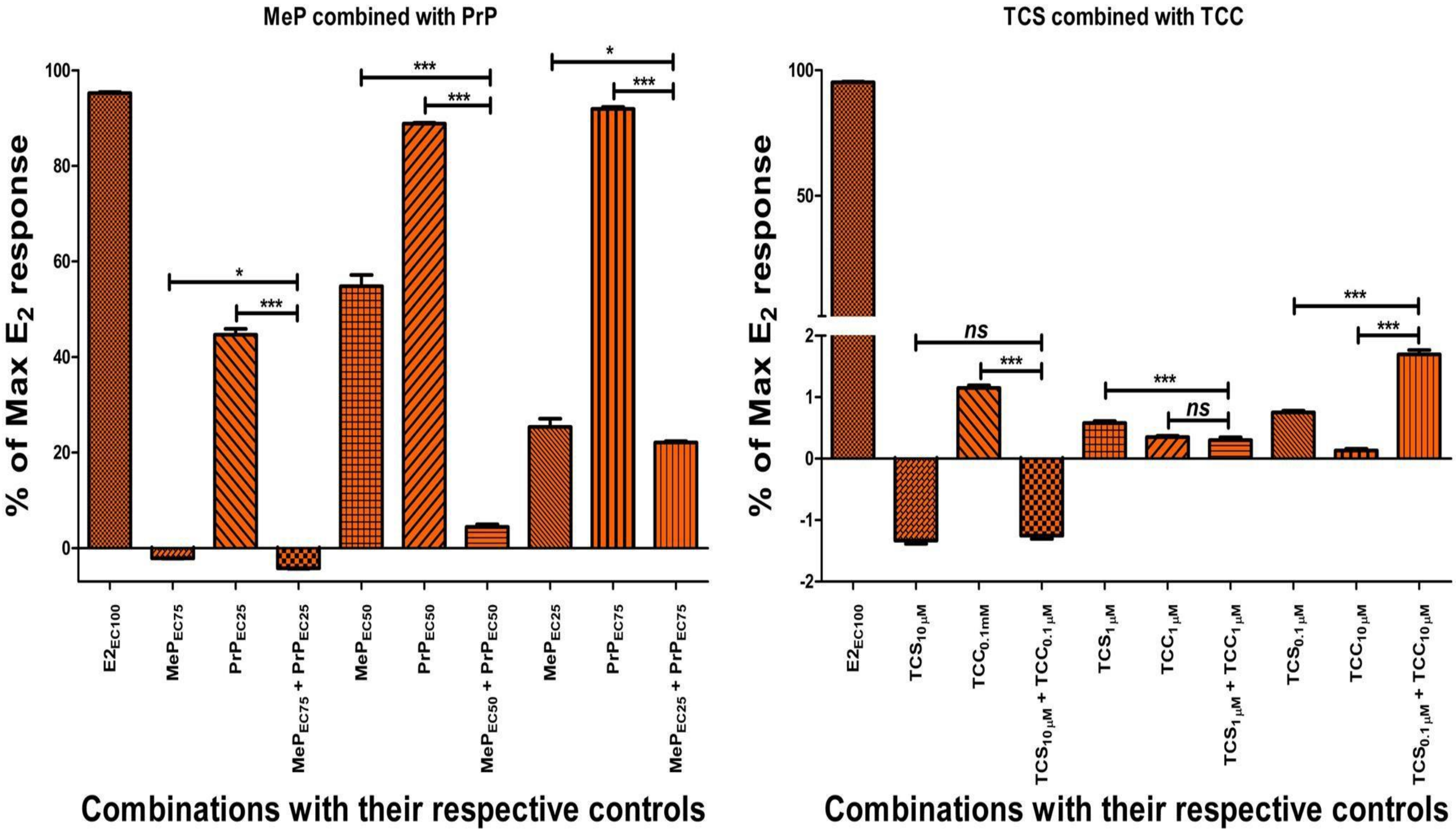

Figure 5.9 ERα modulatory effects observed when combining MeP and PrP or TCS and TCC using the YES. Data shown are represented as the mean ± SE (n = 10), Statistical analysis was performed with one-way ANOVA followed by a Dunnett post-test. Significance are denoted by *, ** and *, corresponding to p < 0.05, p < 0.01 and p < 0.001, respectively. Negative values represent cytotoxic conditions.**

5.3 Discussion

Breast cancer is life changing disease that will affect millions of woman around the world this year. In light of the recent evidence surrounding the presence of EDCs in normal and cancerous breast tissue, as well as breast milk, and their link to ED, this chapter set out to investigate ERα modulatory effects that could originate from their presence. The current chapter made use of potency values calculated in chapter three to perform binary mixture experiments. Combinations between estrogens and MeP, PrP, TCS or TCC were considered as well as combinations between TCS, PrP, TCS and TCC. Results showed additive, synergistic and antagonistic effects between mixtures.

Overall MeP had a more antagonistic effect on mixtures involving estrogens, while additivity was the main combinatory effect observed for co-exposure to PrP. At the time of printing, this is the first report showing 1) the effect on ERα of combination with parabens and estrogens, and 2) two completely different interaction profiles for compounds belonging to the same chemical group. The latter can be of great concern as sharing the same chemical group is often regarded to also having similar physiochemical properties and biological effects. This means that each compound in future studies needs to be investigated for effects such as ED as representatives of a group would not suffice. Furthermore, the antagonistic and additive effects observed indicate that these mixtures could have severe consequences not only to estrogen-responsive BC patients but also woman in general. BC patients would be susceptible to the having additional estrogen-like chemicals present in breast tissue as well as compounds that can either have an additive (PrP) or antagonistic (MeP) effect. However, parabens have been mostly detected in breast tissue as a combination of MeP, PrP, ethylparaben, n-butylparaben and isobutylparaben (341). Results from the current study show that when both MeP and PrP are present, antagonistic behaviours is the norm for their combination. This suggests that combinations of parabens might yield antagonistic behaviour rather than additive. However, a similar study by Charles and Darbre (350) with MeP, PrP, ethylparaben, n-butylparaben and isobutylparaben showed that in combination these five parabens act additively. Nonetheless, findings from Charles and Darbre (350) were obtained using a MCF-7 proliferation assay and therefore conflicting results are a possibility. In contrast to BC patients, the presence of MeP or PrP in woman could have health and developmental effects as their presence together with estrogens could disrupt the delicate key roles estrogens play in woman. In children (E_2 dominating) MeP could hamper the development of secondary sexual characteristics, while PrP would be able to speed up the process. In older woman (E_1 dominating) PrP might aggravate menopausal symptoms, while MeP could possibly rescue woman from these symptoms. Finally, MeP and PrP could disturb the delicate hormone balance in pregnant woman (E_3 dominating) and thereby influence the development of the unborn foetus. Gestation, pre-puberty and puberty are regarded as key windows of susceptibility that can lead to life-long health consequences. Therefore, the use of these compounds in products specifically for pregnant woman, toys for children and other PCP for infants, children and young adults should be carefully considered.

Combinations between TCS or TCC and the estrogens showed antagonism and synergism for the respective EDCs. TCS has been suspected (329, 390) for some time to exhibit anti-proliferative effects in the presence of E_2. In this study results confer the findings of the previous studies. In addition, results also show that the range can now be extended to also include E_1 and E_3. Like TCS, TCC has also been suspected (329–331) to confer the results obtained in this study. Similar to the case with MeP and PrP, the actions of TCS and TCC in combinations with estrogens could have dire consequences. However, the synergistic effects seen with TCC in combination with estrogens could prove more worrisome than that of estrogens combined with PrP. TCC's synergistic action when combined with estrogens makes this compound a number one threat for BC patients battling estrogen-responsive cancers, as the presence of the smallest amount of estrogen and TCC could aggravate the current state of the BC by increasing BC cell proliferation. Finally, the presence of TCC in pregnant woman, pre-pubertal girls and those going through puberty are equally problematic for the same reasons supplied for PrP.

As a last consideration, the interactions between the different EDCs were investigated. First, combinations with TCS and TCC had no noteworthy effects. Second, results showed that TCS could suppress the action of MeP, while TCC had mixed results indicating either antagonism or synergism. The observation for TCS and MeP comes as a relief as its presence in the female body could shield against the actions of MeP when only these two are present. However, this is not always the case as other PCPs such as PrP or TCC is likely to be present. Third, mixed observations were observed for PrP in combination with either TCS or TCC and therefore needs to be confirmed. Finally, of note, the response achieved when 10 µM TCC was combined with either MeP or PrP at their EC_{25} potencies, were more than two times the response of MeP or PrP alone. From this it can be speculated that higher concentrations of TCC could potentially enhance the action of lower concentrations of MeP or PrP and therefore these combinations are a serious concern.

In conclusion, the results obtained from the mixture results indicate a clear modulatory effect on ERα, with some combinations of greater concern than others. However, the findings from this study needs to be confirmed with cell models to further elucidate how the investigated combinations could affect an organism adversely.

5.4 Materials and methods

5.4.1 Materials

Materials used in this chapter are similar to the ones used in chapter 4 for the YES (See section 4.4.1).

5.4.2 Methods

General experimental preparations. Stocks solutions used in this chapter were prepared similar as explained in section 4.4.1. From these the relevant 20x concentrations matching the EC_{25}, EC_{50}, and EC_{75} for E_1, E_2, E_3, MeP and PrP were prepared in MeOH. Stock concentration 20-fold higher than 0.1, 1 and 10 µM was prepared for TCS and TCC. E_2 at a concentration 20-fold 1.5 nM was used as positive control in addition to a standard curve prepared from the 20-fold 1.5 nM E_2. Furthermore, glassware was treated in the same manner as mentioned in section 4.4.1, yeast stored under similar conditions and yeast growth medium kept unchanged as layed out in section 4.4.1, while the experimental procedure was changed minimally.

YES protocol. Yeast cultures where started on day one and passaged as previously mentioned. On day three, 10 µL of each 20x stock of the first compound was plated in combination with 10 µL of the second compound, in sterile 96-well optically flat bottom microtitre plates (n = 12), and air dried. In addition, 10 µL of each 20x stock was also plated separately. MeOH was used as blank, 1.5 nM (20x) E_2 as positive control and the E_2 dilution series as an assay progression indicator. Finally, the assay medium was seeded, plates incubated and readings taken under similar conditions as before.

Statistical analysis. Statistical analysis was carried out with the statistical package GraphPad Prism 5.00, and a one-way ANOVA with Dunnet post-test used to quantify the different effects observed between mixtures and the compounds on their own.

See section 4.4.1 for a detailed description of materials and general assay procedures.

CHAPTER 6

Conclusions and future studies

6.1 Conclusions

This study was part of a larger project that aims to develop decentralised treatment systems, based on biomimicry design, to mitigate the discharge of greywater from informal settlements into the Berg and other rivers. The proposed solution, a natural waste water treatment system called the TJED Eco-machine, showed to be effective in a pilot scale model. However, the actual system still needs to be evaluated at full-scale implementation. This study contributed to this evaluation by setting up methods that can be used to monitor treatment. Three analytical chemistry methods namely GC-, UPLC- and UPC^2-MS/MS were investigated as possible instruments for detecting commonly occurring pollutants in samples taken from the greywater that will be treated in the Eco-machine once installed. The majority of the pollutants selected for monitoring have been shown to play some part in disrupting the endocrine system of mammals and therefore pose a great risk to the environment. The methods developed on the three instruments were optimized and tested for their sensitivity and overall performance towards detecting these pollutants. Method validation showed that the UPC^2-MS/MS had great potential to be used as the instrument of choice for detecting the selected commonly occurring pollutants. However, some of the data suggested that the method needed simplification and further optimization before finally being used as the analytical instrument of choice. In addition to developing analytical techniques for monitoring the levels of pollution and ultimately their removal through treatment systems, studies were performed to show how some of the commonly occurring pollutants selected for monitoring can possibly influence signal transduction in mammals. This is particularly relevant as signal transduction plays a central role in regulating homeostasis. In these studies potency values were determined for the pollutants E_1, E_2, E_3, MeP and PrP with two bioassays (YES and E-SCREEN) that can show ERα modulation. However, the compounds TCS and TCC that was also tested showed no dose-response behaviour. Mixture studies, using the YES, between MeP and PrP, TCS and TCC, MeP and TCS, PrP and TCS, MeP and TCC, PrP and TCC, and between E_1, E_2, E_3 and MeP, PrP, TCS and TCC showed a wide range of mixture interaction. Some were additive, some antagonistic and some synergistic. The most noteworthy and concerning mixtures were that of TCC with E_1, E_2, E_3, MeP and PrP, as TCC showed synergistic behaviour that could greatly influence homeostasis and diseases such as BC if a mixture of these compounds are found in humans and animals. For a detailed overview on the ED effects of the studied compounds and others, chapter 2 section 2.5.5 provides an in depth discussion.

In conclusion, this project fulfilled the overall aim of finding the most suitable chromatography method that could be used to evaluate the JTED Eco-machine‘s ability to remove pollutants such as PPCPs. In addition, the project also fulfilled the aim of showing the possible consequences to humans and animals if exposed to contaminated water resources containing EDCs.

6.2 Future studies

Method developed for isolating, detecting and quantifying pollutants proved to be complex in nature and therefore needs further optimization and simplification. Also, method development failed to include the full spectrum of robustness testing as prescribed by leading authors in the field. Future studies should therefore investigate other variables such as the pH of the eluent, concentration of additives present in the eluent and column batch and age that may influence parameters adversely. Alternatively, variables such as analyte extraction time and the sample transfer process should be investigated for improving process efficiency. On top of that, future studies should also investigate other SPE extraction solvents and SPE equilibration buffer conditions such as salt content.

With regards to mixture studies, results need to be confirmed with a cell model such as the E-SCREEN. Additionally, combinations should also be tested on an ERβ bio-assay as a growing amount of evidence suggests that ERβ suppresses the function of ERα. Alternatively, mixture effects should also be investigated on other NRs considering that there exists a complex interplay between NRs.

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