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Characterising biochemical changes to hepatocellular carcinoma (HepG2) cells upon exposure to green tea extract using untargeted metabolomics

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BSc Forensic Biology & Toxicology and Biomedical Science

This thesis is presented for the Bachelor of Science (Honours) degree in Molecular Biology at Murdoch University, Disciplines of Medical, Molecular and Forensic Science

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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Natasha Louise Morrison

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Abstract

Complementary and alternative medicines (CAMs) have become the preferred medicine for many in replacement of conventional medicines due to cultural or financial reasons. Herbal CAMS, in particular, have become a popular choice of medicine for their purported health benefits. Green tea extract (GTE) contains the major catechins epigallocatechin-3-gallate, epicatechin gallate, epigallocatechin and epicatechin, all of which vary across different GTE products and have become the focus on research into its purported health benefits. However, there have been cases of GTE-induced hepatotoxicity, for which the biochemical pathways have not been characterised. This study elucidates compounds similarities and changes in catechin levels within several different GTE products, and biochemical pathways related to reactive oxygen species (ROS) production affected by acute GTE supplementation in an *in vitro* setting using metabolomic techniques. It was found that GTE hepatotoxicity significantly decreased amino acids, oxoacids and carboxylic acids at 1 mg/mL exposure but produced a different metabolite profile upon 0.1 mg/mL exposure. The results demonstrate that GTE hepatotoxicity is a dose-dependent process that induces ROS production, ATP depletion and apoptosis, which corroborates prior knowledge on this topic. These results utilise a novel field of research, metabolomics, to add insight into the biochemical mechanisms of GTE hepatotoxicity and to observe the mass spectral pattern and levels of four catechins in different GTE products: (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epigallocatechin-3-gallate. This will allow consumers to become more aware of herb-induced liver injury and provide data to aid the regulation of herbal CAMs.

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List of units

µg/µL	microgram per microlitre
µL	microlitre
µM	micromolar
µm	micrometres
µmol/L	micromole per litre
µmol/mL	micromole per millilitre
eV	electron volts
g	grams
h	hour(s)
m/z	mass-to-charge ratio
mg/kg	milligrams per kilogram
mg/mL	milligrams per millilitre
mins	minutes
mL	millilitres
ng/µL	nanograms per microlitres
psi	pound per square inch
rcf	relative centrifugal force
rpm	revolutions per minute
sec	second

Abbreviations

3D	3-dimensional
AAAs	Aromatic amino acids
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
ARTG	Australian Register of Therapeutic Goods
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BCAAs	Branched chain amino acids
Ca^{2+}	Calcium
CAM	Complementary and alternative medicine
CH	(+)-Catechin
CHM	Chinese herbal medicine
CI	Chemical ionisation
CIOMS	Council for International Organizations of Medical Sciences
CO_2	Carbon dioxide
CYP450	Cytochrome P_{450}
DC	Direct current
DMEM	Dulbecco's Modified Eagle Medium
DMEM-/-	DMEM without additives (-/-foetal bovine serum, penicillin-streptomycin and L-glutamine)
DNA	Deoxyribonucleic acid

EBP	Evidence-based practice
EC	(-)-Epicatechin
ECG	(-)-Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin-3-gallate
EI	Electron ionisation
ETC	Electron transport chain
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GSH	Glutathione
GTE	Green tea extract
HCAM	Herbal complementary and alternative medicine
HDI	Herb-drug interaction
HILI	Herb-induced liver injury
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
Mg^{2+}	Magnesium
MMP	Mitochondrial membrane potential
mRNA	Mitochondrial RNA
MS	Mass spectrometry
MSTFA	*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide
MTT	Thiazolyl blue tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NH_3	Ammonia

NHIS	National Health Interview Survey
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
OGG1	8-oxoguanine DNA glycosylase 1
PBS	Pharmaceutical Benefits Scheme
PCA	Principal component analysis
P_i	Inorganic phosphate ion
PLS-DA	Partial least squares-discriminant analysis
PPAR	Peroxisome proliferator-activated receptor
RF	Radio frequency
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSD	Relative standard deviation
RT	Retention time
RUCAM	Roussel Uclaf Causality Assessment Method
SF-DMEM	Foetal bovine serum-free DMEM
SRM	Standard reference material
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TGA	Therapeutic Goods Administration
TGAC	Therapeutic Goods Advertising Code
WHO	World Health Organisation

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1. Literature Review

1.1 Introduction

Medicines have been an important part of human health for many centuries and have revolutionised the way diseases are diagnosed, treated, and managed. One of the oldest known forms of medicine is homeopathy, created by Samuel Hahnemann in 1796.[1] Homeopathy applies the notion of 'natural healing', where diluted substances are administered to mimic symptoms of the disease in belief it will treat it. This ultimately became one of the most popular forms of alternative medicine.[1] Conventional medicines directly contradict Hahnemann's ideology, with the aim to eliminate and eradicate diseases through vaccines and pharmaceutical drugs. Research on conventional medicines has improved quality of life and life expectancy.[2] Furthermore, the pharmaceutical industry in Australia is booming as individuals spent approximately AU$10.8 billion on medicines in 2015-16.[3] The Pharmaceutical Benefits Scheme (PBS) in Australia has made it easier and more affordable to obtain medicines, where eligible people can obtain certain medicines free of charge. Despite easy access to conventional medicines in Australia, many individuals are still turning to complementary and alternative medicines. This brings into question the chemical composition in these medicines and the patient's susceptibility to a toxic reaction.

1.2 Complementary and alternative medicines

Since the 1970s, holistic practices have become more prevalent instead of newly-developed pharmaceutical drugs to treat and maintain human disease.[4] These practices have a variety of names, including: "unorthodox", "integrative", and

"unconventional" medicines, but are most commonly known as complementary and alternative medicines (CAMs). Complementary medicines are those that are used in conjunction with conventional medicines, whereas alternative medicines are those used to replace conventional medicines and which are not normally integrated into a country's health care system.[5] CAMs encompass a range of health practices and consumer products, including: traditional Chinese medicine, Indian Ayurveda medicine, vitamin and dietary supplements, homeopathy, acupuncture, yoga, and spiritual practices.

In Australia alone, the CAM industry has had a growth of AU$2 billion since 2014, which is expected to further increase.[6] The same report by Complementary Medicines Australia indicated that there are approximately 8.1 million consumers who regularly purchase CAMs.[6] Interestingly, there is an increasing CAM usage trend in females aged 45 years old or above, of Caucasian descent, of those who are married, possess tertiary qualifications and have private health insurance.[7-11] Many individuals use CAMs for their acclaimed health benefits, such as: the use of dietary supplements for fat loss, antioxidant properties and brain function improvement. Disturbingly, a significant portion of patients with chronic illnesses, such as cancer,[8,12] gastrointestinal diseases,[13] and respiratory illnesses like asthma,[14] turn to CAM for treatment or management of their disease in preference to professional medical advice. This may be due to cultural beliefs, distrust or dissatisfaction with current conventional medicines, and the feeling of 'taking control' of their treatment.[8,12,15]

Although there is evidence that pharmacists and physicians are a primary source of information for some individuals,[8,13] a recent meta-analysis found that only

approximately 33% of consumers report CAM use to their practitioner.[16] Common reasons for this include: fear of judgement from physician; lack of knowledge from physician; and, alarmingly, the physician not asking during initial assessment.[16] Physicians feel that they should be more educated on the most prominent CAMs[17] and should communicate this with their patients as the history of medication usage, including CAMs, is paramount in determining causality for disease. A portion of physicians and pharmacists, not involved in practicing naturopathy, believe that CAM should be incorporated into conventional practices. This is to provide the best outcome and satisfaction for the patient, despite expressing fears of inadequate safety regulations.[15,18] Regardless, consumers still choose CAMs over conventional medicines for reasons other than wellness. Up to 54% of consumers in under-developed countries use CAMs as conventional medicines are too expensive.[12] Despite their proposed health benefits, some of which are yet to be scientifically validated, the use of CAM remains controversial.

1.2.1 Herbal CAMs

Herbal CAMs (HCAMs), by definition, are complex active ingredients from plants or plant-derived products that are used for medicinal purposes[5] and have been used in traditional medical practices for centuries. An example of a popular HCAM that has been used consistently in the past, and still used today, are the Chinese herbal medicines (CHMs). These CHMs consist of a complex mixture of herbs, not always from plant products, and are used as a 'cure' for any imbalance of energy forces.[5]

HCAMs generated up to AU$690 million in sales in 2017.[6] A 2009 report from the National Health Interview Survey (NHIS) found that HCAMs accounted for

approximately 43.7% of all CAM sales in the USA, making them one of the most commonly consumed CAM.[19] The purchasing of HCAMs has become less challenging as they can be readily purchased through supermarkets, pharmacies, and the internet,[6] which contributes towards such staggering sales figures. While research into HCAMs has increased over the last decade, the molecular complexity of many HCAMs remains unknown. This, combined with the ease in acquiring these products,[6,13] has led to concerns by researchers about the efficacy, safety and appropriate regulation of these medicines.

1.3 Regulation of CAMs

The World Health Organisation (WHO) first introduced the "WHO Traditional Medicine Strategy" in 2002 with the intention of improving the safety, efficacy and quality of CAMs worldwide. The WHO recently updated their strategy and introduced the "WHO Traditional Medicine Strategy 2014-2023" to combat challenges experienced by many WHO members, such as: the enforcement of the policies outlined by the strategy; effective education about CAMs to physicians, and most importantly, the development of a standard assessment for the efficacy, safety and quality of CAMs.[5] In their initiative, the WHO has acknowledged that there must be evidence in order to integrate CAMs into conventional health care.[5] As such, there is a push towards the development of 'evidence-based practice' (EBP) policies for CAMs; regulatory bodies across the world share a common goal of protecting the consumer and, justifiably, many believe that ensuring the safety of CAMs that are clinically relevant are more important than investigating efficacy.[20]

The pressure to employ EBP in CAM research has been met with criticism from philosophers and those with traditional cultural beliefs. Despite a large abundance of randomised controlled trials on CAMs, there is weak evidence towards positive clinical effects of CAM, and some have claimed that there is bias towards publication of positive results.[21] The source of information about CAMs that consumers gather can influence their decision on choosing certain treatments,[13] and bias in published research can provide consumers with undue optimism. Consumers may view these ambiguous positive results as a quicker and cheaper fix to their illness(es), which can be detrimental for their health and to a researcher's reputation.

In addition, the availability of broader choice in the health care system creates potential ethical dilemmas.[22] Whether it be for financial, economic or cultural reasons, many have no alternative but to use CAMs in conjunction with or in place of conventional medicines. This seems to perpetuate an unequal health care system where, in general, those with higher incomes in Western societies have access to safer and more efficacious treatments. It has therefore been questioned whether the regulation of CAMs should be left up to governing public health bodies,[22] as there is evidence that consumers seek to educate themselves about CAMs and their safety.[8,13] However, the integration of CAMs into conventional health care systems requires rigorous changes to current legal and regulatory systems.[23] Thus, those that are disadvantaged may be concerned about harsher regulatory practices and legalisation for fear of losing access to CAMs.

1.3.1 Regulation in Australia

The Australian Government passed the *Therapeutic Goods Act 1989* to manage and secure the manufacture, exportation, importation, supply and testing of CAMs. All CAMs and other therapeutic goods are regulated by the Therapeutic Goods Administration (TGA), which is a division of the Department of Health. Currently, CAMs are divided into two separate categories using a risk management assessment of ingredients: "registered" medicines, which include prescription medicines and many over-the-counter medicines; and "listed" medicines (AUST R and AUST L labels, respectively).[24] The key differences between registered and listed medicines are that: efficacy is not evaluated by the TGA for listed goods, and goods solely for exportation are exempt from being registered.[24] If approved, medicines are added to the Australian Register of Therapeutic Goods (ARTG), a database showing all therapeutic goods that are registered, listed, and under review.

In Australia, HCAMs and their specific ingredients, such as herbal extracts, can also be categorised either as listed or registered medicines on the ARTG. The TGA released a guidance document in 2011 outlining herbal extracts that are equivalent to ingredients currently approved by the TGA, and thus may be used as an alternative ingredient in the manufacture of therapeutic goods.[25] Factors such as geographical location and solvent used in the extraction of herbs can influence the extraction profile and, therefore, variation in batches occur.[25] This document outlines which parameters can influence the native extract ratio (the ratio of the mass of the herbal material to the resulting material extracted from the herbal plant) and will help develop standard extraction protocols approved by the TGA.

Although Australia has one of the most rigorous regulatory systems in the world, the framework has been met with criticism. In particular, there have been numerous concerns raised to the Complaints Resolution Panel regarding the compliance of advertisements in relation to the Therapeutic Goods Advertising Code (TGAC), primarily with the pre-approval process for advertisements. The TGAC prevents misleading information being presented in advertisements and requires all medical benefits and risks be accurately portrayed.[26] Failure to comply with the TGAC could result in the manufacturers receiving up to five years imprisonment or 4,000 penalty units (a fine of approximately AU$645,000), or both.[26]

1.3.2 Efficacy and safety concerns

Concerns persist regarding the efficacy and safety of CAMs, primarily due to limited research. Of those studies that have been published, many did not apply EBPs, resulting in controversy and conflict between CAM and conventional medicine users. Tanaka and colleagues[27] theorised a mathematical model that demonstrates that the most efficacious treatments with strict requirements for use may not always become popular and widespread compared to other ineffective treatments, such as CAMs. They argue that ineffective treatments can prolong illness time and therefore elevate purchase of these inefficacious treatments. However, some medical researchers have argued that the efficacy of evidence-based conventional medicines is also of concern. This has been proposed to be due to a growing concern about physicians being unaware of evidence-based medical guidelines,[28] which can affect the administration of appropriate medication to disease sufferers. Strategies such as gaining more access to the guidelines and

seminars to improve on knowledge have been implemented that has improved this particular issue.[29]

With the growth in CAM sales, there has been greater concern amongst health practitioners as to the safety of CAMs. There are a variety of reasons for this increase in concern, including: lack of regulation, as previously discussed; lack of incident reporting, and the belief of CAMs being 'natural' and 'safe'.[30] A major concern is the potential for CAMs to interact or interfere with conventional medicines. Several reviews have not found compelling evidence or cases of such interaction with clinical significance,[31,32] which could be due to the fact that potentially hazardous interactions are rare.[33]

1.3.2.1 Safety concerns for HCAMs

As with all CAMs, safety concerns with HCAMs arise due to a number of reasons, including:

- Inadequate research data, funding for research, and support for research;
- Lack of education and/or training, including health practitioners; and
- Lack of expertise on HCAMs within public health authorities, and other relevant bodies.[5,34]

The combination of these issues across many countries heightens the concern of HCAM safety; the lack of research data reduces the effectiveness of laws integrated into a public health system and could lead to malpractice or adulteration of traditional herbal preparations. For example, there have been cases where several herbal remedies were identified to have potentially fatal toxic metal concentrations, possibly as a result of inadequate quality control checks.[35]

Additionally, researchers are beginning to focus their research in herb-drug interaction (HDI), defined as complex components of HCAMs that interact with other conventional drugs to up- or down-regulate their actions. The chemical complexity of HCAMs can make research into understanding and characterising HDIs difficult. Thus, few studies have shown promising results of HDI of clinical relevance,[11,36] but clinical cases and randomised clinical trials still exist.[37] One such HCAM, green tea extract (GTE), has been the centre of attention for approximately 35% cases of potential HDI in recent years.[11] This highlights the significance of GTE as a desirable herbal extract for research to clarify its safety and toxic potential.

1.4 Green tea extract

Green tea, obtained from the *Camellia sinensis* plant, is one of the most popular beverages in the world.[38] Besides its brewed form, green tea can be consumed in extract form, which has led to its use as a popular dietary supplement. GTE is a popular dietary supplement taken in the form of a pill, powder or liquid. It differs from its brewed form such that an extract is synthesised using a non-alcoholic solvent to remove or select for certain compounds from green tea, therefore making it more concentrated.[25] Its popularity has meant that more research is being conducted to investigate its therapeutic efficacy, including: fat reduction,[39] anti-cancer effects,[40,41] and improvement of cardiovascular health.[42]

The purported benefits of GTE have been linked to the presence of flavonoids, a sub-category of polyphenols. Flavonoids are naturally occurring, biologically active, organic compounds found in many foods and plants. GTE contains a sub-category of flavonoids known as the flavan-3-ols, and the four major flavan-3-ols

in GTE are: (-)-epicatechin (EC); (-)-epigallocatechin (EGC); (-)-epicatechin gallate (ECG), and (-)-epigallocatechin-3-gallate (EGCG) (Figure 1.1). Collectively, these compounds are known as the catechins.[43] EGC is the hydroxyl derivative of EC, whereas ECG and EGCG are the gallic acid derivatives of these two compounds.

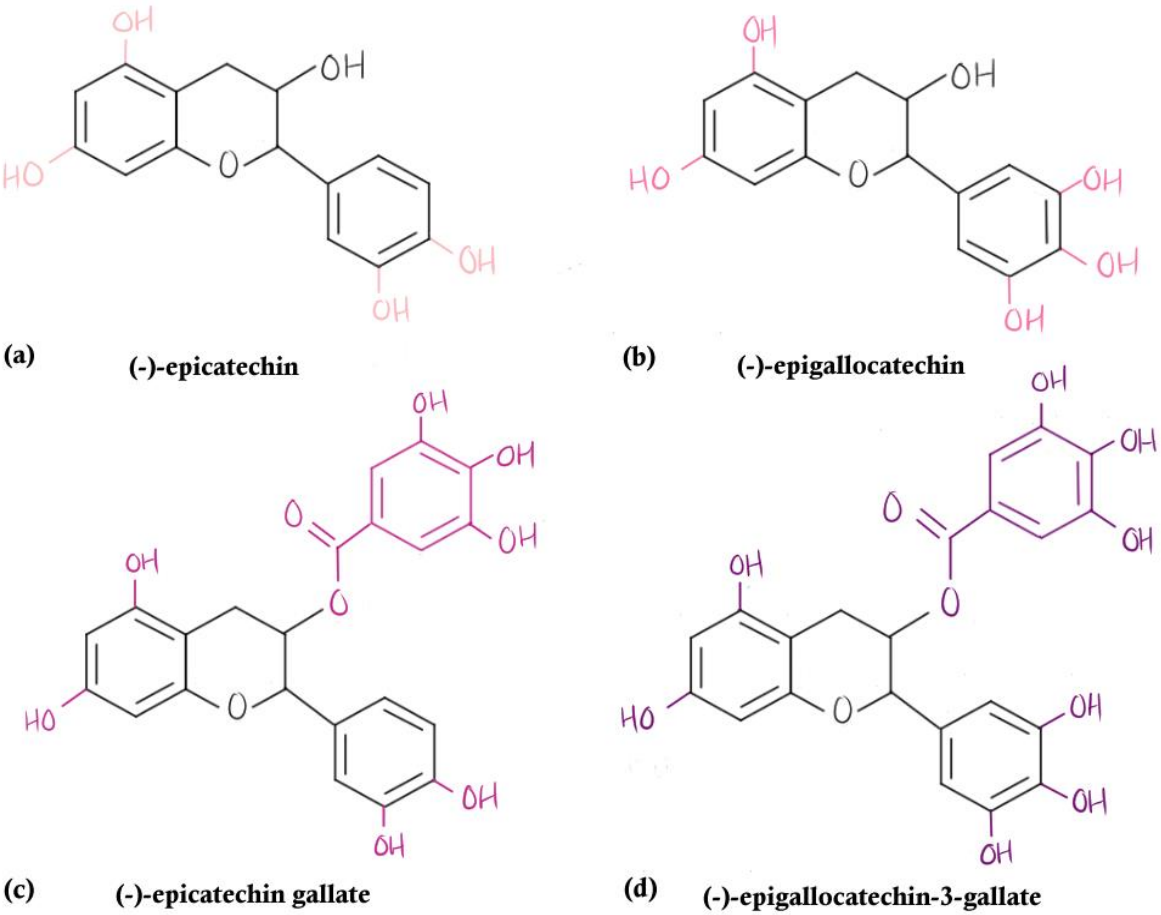

Figure 1.1. Molecular structures for the major catechins. (**a**) EC; (**b**) EGC; (**c**) ECG, and (**d**) EGCG. The flavan-3-ol backbone structure is highlighted in black.[46] Variations in molecular structure are shown using different colours.

1.4.1 Catechins

Catechins are found in a number of food products, such as black tea, apples, red wine, dark chocolate and berries. The catechin dry weight in GTE can reach up

to 42% and varies depending on the season and growing location.[43] As catechins comprise the majority of the complex mixture in GTE, they have been the main focus of research into both health benefits and potential toxicity. The catechins play a major role as antioxidants; specifically, they are involved in the clearance of over-saturated free radicals, such as reactive oxygen (ROS) and nitrogen species (RNS). By oxidising these free radicals that would otherwise lead to oxidative stress, the catechins prevent cellular and genetic damage. The low electrochemical potential of flavonoids, including catechins, increases free radical scavenging ability, creating a stronger antioxidant response.[44] The high catechin content increases the antioxidant power of GTE, strengthening the perception that GTE is a highly valuable when integrated into the diet.

The chemical structure of catechins consists of a general flavan-3-ol backbone, with a molecular formula of $C_{15}H_{14}O_2$.[45,46] Variations of this general structure occur to form EC, EGC, ECG and EGCG, with the addition of a gallic acid at the C_3 position for ECG and EGCG (Figure 1.1). The biosynthesis of EC and EGC begins with phenylalanine and involves many enzymes of different functions, such as reductases and hydroxylases, to form cyanidin. This precursor, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), undergoes an important redox reaction with anthocyanidin reductase to form EC, and can further undergo a transferase reaction to add another hydroxyl to form EGC. A gallate is added to EC and EGC using flavan-3-ol gallate synthase to form ECG and EGCG, respectively. Catechin content can vary depending on the species, growing location, developmental stage[47] and availability of light,[48] all of which may affect the quality of GTE products. This variability further contributes to the growing concern of consumption safety for HCAMs.

One study suggests that catechins with gallic acid moieties (ECG and EGCG) have a higher potential to cause hepatotoxicity than EC and EGC in primary rat hepatocytes.[38] Interestingly, another study found that the non-gallated molecules were mostly present in conjugated forms, whereas the gallated molecules were mostly present in free form in plasma.[49] Plasma levels were heightened when human volunteers were fasting, thus ultimately increasing the bioavailability of the green tea catechins. These studies suggest that increased bioavailability, the molecular structure and the moiety of green tea catechins may induce GTE-related hepatotoxicity.

1.5 The liver and hepatotoxicity

Along with biliary and Kupffer cells, hepatocytes form the primary structure of the liver. Hepatocytes are the major functional cells of the liver which carry out the important task of filtering the blood for macromolecules, amino acids, fatty acids, toxins and other foreign compounds from the digestive tract, as well as transporting the filtered blood to the rest of the body. The liver undertakes other essential bodily functions such as protein synthesis, metabolism, detoxification and biotransformation of molecules.

1.5.1 Liver metabolism

While most organs and tissues possess the ability to biotransform various xenobiotics, such as drugs and alcohol, the most central site is the liver. A xenobiotic typically undergoes Phase I and Phase II metabolism. Phase I metabolism modifies the pharmacological activity of the xenobiotics and its metabolites. These reactions include reduction, oxidation, hydrolysis and

deamination, which is often catalysed by enzymes such as isoforms of cytochrome P_{450} (CYP450).[50] This is followed by Phase II, which is biotransformation into a more polar substance for excretion through the kidneys. Common conjugation reactions that occur include methylation, glucuronidation, sulfation and acetylation. This process does not require the use of CYP450 as a catalyst, but instead uses transferases. Xenobiotics may also undergo Phase III metabolism, which uses transporters in the liver to export the inactive xenobiotic and its metabolites out of the liver and to the kidney for excretion via urine.[50] The products of xenobiotic metabolism can also build up and can be detrimental for liver health, which may be a contributing factor in GTE hepatotoxicity.

1.5.2 GTE hepatotoxicity

Although researchers have suggested a possible dosage level for GTE hepatotoxicity *in vivo* and *in vitro*,[38,51,52] it is an idiosyncratic disease, meaning it can be unpredictable in terms of the dosage and an individual's molecular response. The rarity of GTE hepatotoxicity cases has made it difficult to determine the median lethal dose threshold (LD_{50}) in humans, suggesting that it could be due to genetic diversity within the population that causes degrees of susceptibility to liver injury. Metabolic capacity also varies among individuals.

Some individuals may metabolise xenobiotics rapidly and not experience a therapeutic effect, while others may have a toxic effect even at low doses due to slow metabolism. These are respectively known as 'slow' and 'fast acetylators', with differing arylamine *N*-acetyltransferase (NAT) enzyme activities due to polymorphisms of the *NAT1* and *NAT2* genes.[53] Polymorphisms of these genes that down-regulate activity are generally seen in those of Caucasian descent.[53] In

addition to the NAT enzymes, polymorphisms in the CYP450 gene pool also contribute to an individual's response to xenobiotics, where particular isoenzymes have been identified as most significant.[54] GTE hepatotoxicity has been demonstrated in genetically diverse animal models, with some mice tolerating exposure to GTE and some experiencing severe hepatotoxicity.[55] Recent research suggests that the catechins in GTE can also affect functioning of some Phase I CYP450 isoenzymes, such as significantly inducing CYP1A1[56] and CYP3A4 activity[57] in the liver. Other studies showed that exposure to GTE had little to no effect on the functioning of the CYP450 isoenzymes, although have suggested that it inhibits CYP3A4 activity.[58,59] This conflicting information enforces the idiosyncratic nature of GTE hepatotoxicity and results in significant challenges determining doses required for a toxic effect. The range of CYP450 isoenzymes that GTE up- or down-regulates could also affect measurement or characterisation of the biochemical mechanism of GTE hepatotoxicity.

Available literature suggests that the biochemical mechanism of GTE hepatotoxicity is not fully understood. Furthermore, it is also not clear in which form the components of GTE induce their cytotoxic action. Studies to date have used liver function tests, biochemical assays and clinical symptoms to observe the end-point effects of the toxin. In addition, the diversity of xenobiotic metabolism rates within the population makes it difficult to observe GTE toxicity. There have been cases in recent years of hepatotoxicity suspected to be triggered by GTE,[60-63] leading to a phenomenon known as herb-induced liver injury (HILI). Interest into researching GTE hepatotoxicity gained traction after cases of hepatotoxicity involving the weight loss supplement Exolise were published.[64] Upon review, Exolise was discontinued from the market in France and Spain in 2003. The

available research conducted thus far has suggested that the catechins are likely involved in hepatotoxicity. With evidence of cases of HDI[11] and hepatotoxicity involving GTE, it is paramount to identify the most likely native catechin(s) involved in disease and elucidate the biochemical pathways that may contribute.

1.5.2.1 Green tea catechins and hepatotoxicity

The available literature have attributed the hepatotoxic effects of GTE to the catechins and their potential pro-oxidant effects, namely increasing ROS production, particularly with high doses of EGCG.[38,65] Excess ROS can be detrimental to cellular health; it can cause damage to the cell membrane, resulting in increased permeability and allowing toxins into the cell. GTE can also induce double base lesions in cellular and isolated deoxyribonucleic acid (DNA), accelerated in the presence of metal ions,[66] which can result in mutations. Most importantly, the catechins have been found to collapse mitochondrial membrane potential (MMP).[38] The MMP is an intermediate process formed from the production of the proton gradient during adenosine triphosphate (ATP) synthesis within cells. A drop in the MMP can lead to stunted ATP synthesis and has also been suggested to induce programmed cell death (apoptosis).[67] However, the biochemical reasoning behind induction of oxidative stress has not been characterised in the literature. More specifically, it is poorly understood whether it is due to the accumulation of native GTE components or its metabolites.

EGCG and ECG are typically the most abundant catechins, followed by EGC and then EC.[68] EC abundance is low, ranging from 2-4% of the total catechin content,[68] but this does not limit its potential as a health benefit. The most popular use of EC is for improving skeletal muscle health. Researchers have claimed that, at low

doses, EC can suppress myostatin to prevent inhibition of skeletal muscle growth,[69] and it has already been adopted by bodybuilders and those affected by muscular illnesses. While side effects are not well understood, there is no evidence to suggest that EC alone plays a role in hepatotoxicity.[38,52] This could be due to the low relative abundance of EC in GTE, which may affect the overall dosage required to have a significant hepatotoxic effect. EGC has a greater potential to cause oxidative damage and mitochondrial membrane instability in hepatocytes than EC,[38] but not enough to sustain a lethal cytotoxic response,[52] suggesting that the gallic acid moiety may be involved in hepatotoxicity.[38]

ECG is similar to EGCG in that they both possess a gallic acid moiety and it is only second to EGCG in inducing a hepatotoxic response.[38] However, ECG has not been as extensively researched as EGCG.[68] EGCG is known for its purported health benefits, and, as such, has been widely researched for its anti-cancer[41] and weight loss properties through its antioxidant function.[70] Although sometimes beneficial, its higher abundance in GTE has made EGCG the target for research into both acute and chronic hepatotoxicity. Lambert and colleagues[51] attributed EGCG to dose-dependent hepatotoxic responses *in vivo*, which has also been demonstrated at high doses *in vitro*.[65] Lambert showed that continuous oral doses of EGCG above 750 mg/kg can potentially cause irreversible damage to murine hepatocytes *in vivo*.[51] Galati et al. showed that glutathione (GSH), a potent antioxidant involved in clearing damaging oxidants, decreased in the presence of EGCG, which contributed to increased ROS production.[38] However, it is difficult to assess the toxicity of complex mixtures in humans, such as those in GTE, compared with purified molecules due to various synergistic interactions and varying bioavailability.[71]

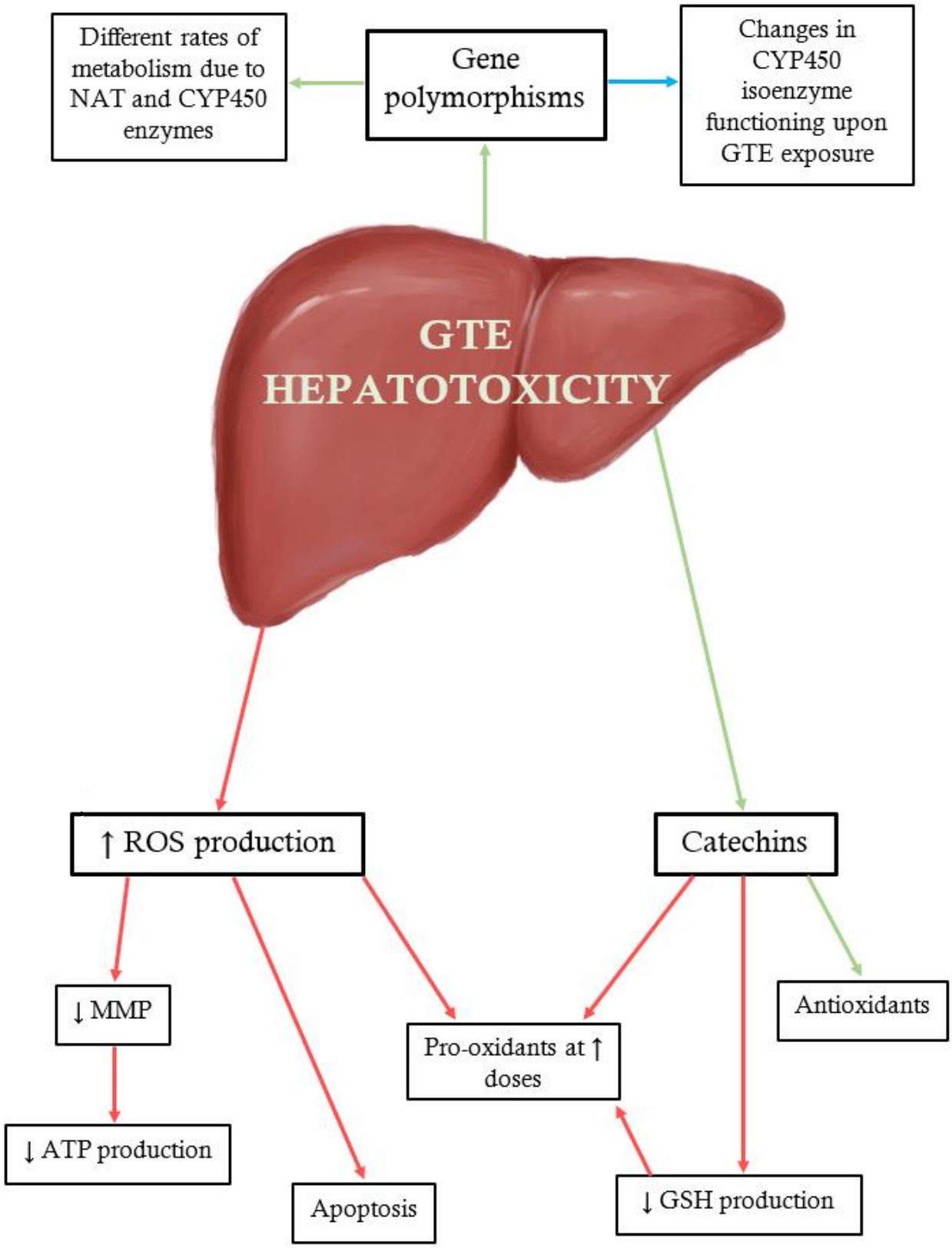

Figure 1.2. Summary of hypothesised means of GTE hepatotoxicity. Key: green = elucidated; red = inconclusive information from the literature; blue = conflicting information from the literature.

The available evidence identifies EGCG as the most probable catechin to cause the most severe hepatotoxic effects both *in vitro* and *in vivo*. This is possibly due to its high abundance and antioxidant activity, which could induce an acute overdose in humans with a single dose of GTE. These studies have used animal models and clinical biochemistry assays to characterise hepatotoxicity of GTE. However, no suggestions have been made as to the biochemical pathway(s) affected by GTE and its catechins within human models, and so the exact pathways involved remain inconclusive. The hypothesised avenues of GTE hepatotoxicity are summarised in Figure 1.2. As GTE consumption is popular, a summary of the biochemical pathway(s) it modulates is required to determine if it is clinically relevant for consumers.

1.5.3 Cases studies into GTE hepatotoxicity

As the popularity of GTE has increased,[4] there have been more reported cases of HILI related to GTE. A selection of these cases and clinical characteristics are summarised here, chosen on the basis of different individual responses and incidences reported most recently.

Surapaneni and colleagues[60] reported a case of suspected acute hepatocellular injury in the USA in 2017. A 50-year-old Caucasian woman presented with symptoms of weakness, decreased appetite, night sweats, severe itching, and jaundice-like symptoms. Liver function tests showed that the patient was experiencing elevated levels of total and direct bilirubin, aspartate aminotransferase (AST) and alanine transaminase (ALT). Clinical tests related to

liver illnesses such as hepatitis and herpes were negative. A liver biopsy showed enlargement of the hepatocytes (ballooning degeneration), confirming that hepatic necrosis had occurred. The patient revealed that they had been taking supplements that contained GTE once a day for one month. The authors concluded that GTE may have been the cause of severe acute hepatotoxicity using a Roussel Uclaf Causality Assessment Method (RUCAM), a point-based assessment method to determine the most likely cause of liver toxicity based on numerous factors.

A case reported in 2013[61] showed a 16-year-old American Hispanic male who presented with jaundice-like symptoms. AST, ALT, conjugated bilirubin and unconjugated bilirubin were all significantly above normal reference ranges. Hepatitis and other viral tests were reported to be negative. The patient disclosed that they had a history of obesity and were taking a concoction of dietary supplements, including GTE. The patient was taking two pills per day for 60 days prior to admission to hospital, equivalent to 400 mg EGCG per day. A liver biopsy showed ballooning degeneration and cellular necrosis, similar to the previous case. The patient was given ursodiol and vitamin K and liver function tests gradually reverted to normal. The authors concluded that the herbal supplement was the most likely cause of liver failure, however, this was not determined by the RUCAM method or similar.

A more serious case of hepatotoxicity was reported by Whitsett and colleagues.[63] In 2013, a 52-year-old American woman of unknown ethnicity presented to hospital with jaundice-like symptoms, vomiting and mild abdominal distension. The patient admitted to using GTE for two days whilst fasting. Total bilirubin, AST and ALT were all highly elevated, while hepatitis and other viral tests were

negative. A CT scan showed fluid build-up in the abdomen and a nodular liver. After a course of corticosteroids, the patient returned to hospital with worsened symptoms of hand tremoring (asterixis) and slurred speech, indicating that liver injury had progressed. Two days later, the patient underwent a liver transplant. A biopsy of the diseased liver was taken, with central cellular necrosis showing that hepatotoxicity had occurred most likely due to GTE. Again, the RUCAM was not applied in this conclusion.

Another serious case occurred in Perth, Western Australia in 2015.[62] A 26-year-old Indigenous Australian male presented with jaundice-like symptoms and fatigue. Ten weeks prior to admission the patient had used two different dietary supplements for one week and discontinued use after developing rigors. Clinical liver function tests were conducted, and ALT, AST and bilirubin levels were severely elevated. The patient's condition worsened, and he developed asterixis, whilst albumin depleted below the reference interval. A liver biopsy was taken and showed cellular necrosis. The patient underwent a liver transplant two months after initial presentation to hospital. The diseased liver was taken for histopathological testing and showed disorganised organ architecture with necrosis. Although more than one dietary supplement was used, the authors concluded that the most probable cause of this severe hepatotoxicity was GTE. The Council for International Organizations of Medical Sciences (CIOMS) scale was used in place of the RUCAM to come to this conclusion.

Table 1.1. Summary of four cases of GTE hepatotoxicity. The age, sex, symptoms, biological tests, histopathology, and assessment method are noted. N/A = not available.

Case	Age (yrs)/Sex	Symptoms	Tests	Histopathology	Assessment Method
Surapaneni[60]	50/F	Weakness, ↓ appetite, night sweats, itching, jaundice-like symptoms	↑ Total and direct bilirubin ↑ AST and ALT	Ballooning degeneration	RUCAM
Patel[61]	16/M	Jaundice-like symptoms	↑ Conjugated and unconjugated bilirubin ↑ AST and ALT	Ballooning degeneration Necrosis	N/A
Whitsett[63]	52/F	Jaundice-like symptoms, vomiting, abdominal distension, slurred speech, asterixis	↑ Total bilirubin ↑ AST and ALT	Central cellular necrosis Required transplant	N/A
Smith[62]	26/M	Jaundice-like symptoms, fatigue, asterixis	↑ Total bilirubin ↑ AST and ALT ↓ albumin	Disorganised liver architecture Necrosis Required transplant	CIOMS

A summary of the cases can be found in Table 1.1. The cases involved patients from different ethnic backgrounds, ages and genders, showing that the dangers of GTE-containing products are not limited to specific groups. Results of the liver function tests were similar in all GTE hepatotoxicity cases, although alkaline phosphatase (ALP) levels were either normal or slightly elevated compared to the rest. This feature may rule out biliary cirrhosis and cholestasis,[72] however, it is unlikely to be unique to GTE hepatotoxicity. The most common physical symptoms were the jaundice-like symptoms, with two of these cases progressing to worsened physical symptoms like asterixis, resulting in liver transplantation. This arises as a complication of a patient's severe hepatic disease, which results in stunting the liver's normal metabolic function and, therefore, build-up of toxic native molecules and/or its metabolites within the spinal cord and brain.[73]

The histological, symptomatic and clinical features of GTE hepatotoxicity have been summarised and correlate to cellular necrosis of the liver, which is non-specific to GTE hepatotoxicity and can be found in other liver diseases. These effects could be exacerbated for those with already damaged livers, such as heavy drinkers and obese patients.[74] There are features, such as normal ALP levels and asterixis, that are conspicuous and potentially useful to medical practitioners. A new approach is required to characterise the biochemical mechanism of GTE hepatotoxicity. A modern and developing field of research, metabolomics, could be the key to characterising this biochemical mechanism.

1.6 Analytical techniques

Although initially used for *in vivo* studies, metabolomics has become increasingly valuable for *in vitro* hepatotoxicity studies to mirror single organ toxicity studies in

human cell lines.[75] The switch to *in vitro* studies has largely been due to the publication of 'The Principles of Humane Experimental Technique' by Russell and Burch in 1959. The three main aims of this document were the: replacement of animals with other methods; reduction in the number of animals used, and; refinement of techniques used to minimise the impact on animals.[76]

In vitro hepatotoxicity studies use a variety of different cell types, such as primary human hepatocytes, human hepatoma cell lines, and adult stem cells. The human hepatocellular carcinoma cell line, HepG2, is one of the most popular cell models in human hepatotoxicity studies;[75,77] it is a commercially available product that has a low cost, low biological variability and high reproducibility.[77,78] This is in contrast to primary human hepatocytes, where functionality and life span is diminished over time when used *in vitro*,[77] and thus not suitable for chronic hepatotoxicity studies.

1.6.1 Metabolomics

Metabolites are molecules of low molecular weight formed during metabolism and are necessary for driving essential cellular processes. They encompass a range of different molecules, including lipids, peptides and nucleic acids. Metabolites are influenced by both genetic and environmental stimuli, which results in a specific phenotype at a point in time.[79] However, the complex nature of metabolism has made it challenging the metabolome to investigate due to transcriptional and post-translational mechanisms, and protein-protein interaction networks required for functioning.[80] Furthermore, metabolites vary in physicochemical properties such as polarity, solubility, stability and size, thus requiring varied analytical approaches.[81]

Metabolomics, the study of the metabolites formed within a biological sample (the metabolome), attempts to address this problem and has detected an increasing number of metabolites over time.[82] This novel field of research began to gain traction in the 2010s.[77] Other 'omics' approaches include genomics, transcriptomics and proteomics (Figure 1.3). Metabolomics is highly useful and differs from other omics fields in that it: (i) represents the final downstream phenotype of an organism to improve the link between cellular pathways and their biological mechanism, (ii) allows observation of abnormal changes in biochemistry before damage occurs,[83] and (iii) brings a holistic approach to biochemical pathway and disease toxicity analysis compared to single marker techniques, such as assays. Therefore, metabolomics is a useful area of research for evaluating biomarkers of toxicity and mapping the affected pathways.

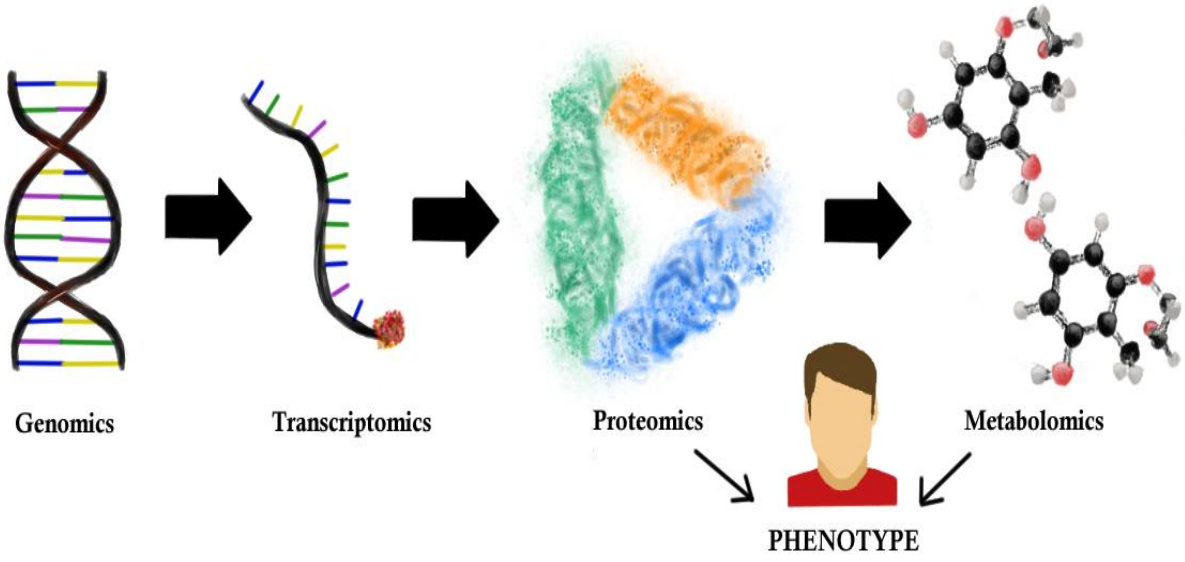

Figure 1.3. The 'omics' cascade in successive order. Proteomics and metabolomics represent the phenotype of an individual.

Metabolomics can be classified into targeted and untargeted analyses. Targeted analysis enhances sensitivity and selectivity to identify and quantify known metabolites of a sample. Whereas untargeted analysis can identify a broad scope of metabolites where prior knowledge of the metabolome for the sample is not required.[79] Untargeted analysis is an excellent tool for the discovery of thousands of metabolites, and is most commonly analysed using a chromatography technique.

1.6.2 Gas chromatography

Chromatography allows for the separation of individual metabolites in a sample mixture by exploiting the ability of different compounds to interact with a stationary phase.[84] The most commonly used chromatography techniques in metabolomics and toxicology are liquid (LC) and gas chromatography (GC).

GC is one of the most widely used separation techniques in a variety of disciplines, including metabolomics. Generally, GC is the method of choice for the separation of volatile substances, such as non-polar molecules. Before analysis of non-volatile molecules can occur, the sample must be derivatised, typically using *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), which improves thermostability and increase volatility.[84,85] In this way, derivatisation improves resolution of metabolites within a sample.[85] The sample is introduced to the chromatogram inlet with a heated sample port to vaporise it. It is then mixed with the mobile phase, which is usually an inert gas such as helium or nitrogen. The mobile phase carries the sample mixture directly through a coiled column with contains the stationary phase. The stationary phase is either an inert liquid (termed gas-liquid chromatography) or a solid non-volatile substance (termed gas-solid

chromatography).[84] Separated compounds are detected by a detector, which reveals peaks corresponding to certain compounds (the chromatogram)[86] (Figure 1.4).

Separation of the sample mixture is based predominantly on the vapour pressure of the compounds. For example, a compound that has been readily vaporised has a higher vapour pressure, which increases its elution or retention time. The opposite occurs for a compound that has a lower vapour pressure and tends to interact more with the stationary phase (Figure 1.4). Separation is also dependent on other factors, such as polarity. Different compounds with similar vapour pressures can be detected based on the strength of intermolecular interactions between the compound and stationary phases, improving chromatographic resolution. Many factors are considered when optimising the column for separation, such as polarity of the sample and stationary phase, and thickness of the stationary phase.[85]

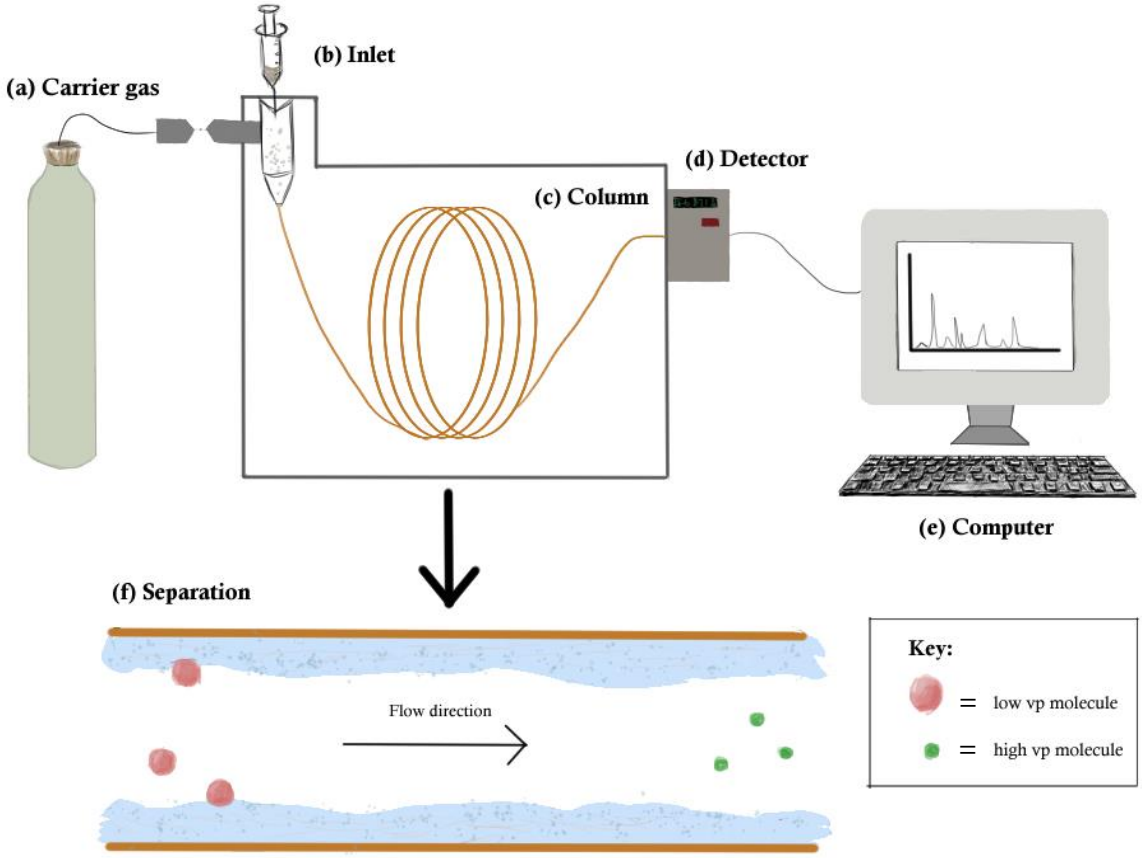

Figure 1.4. Schematic of GC workflow. (**a**) The carrier gas enters the inlet (**b**) and the heated inlet vaporises the sample being injected. (**c**) The gas carrying the vaporised sample enters the column for separation based on vapour pressure, illustrated in (**f**). (**d**) The sample molecules are measured using a detector. (**e**) The chromatogram is displayed on the computer. Molecules with a low vapour pressure (vp) (red) move slower in the column than those of higher vp (green).

Gas chromatography-mass spectrometry (GC-MS) has been a well-established and standardised method of choice for the discovery of a variety of metabolites.[87] It is regarded as the 'gold standard' in metabolomics as it has high chromatographic resolution, sensitivity and reproducibility.[88] Furthermore, the metabolomic databases for GC are more extensive than those for LC.[82] The coupling of GC to MS has been used in *in vitro* hepatotoxicity studies in 70% of the literature,[77] reinforcing its use as a 'gold standard' method. However, the extra step of chemical derivatisation of an analyte can introduce problems in metabolite

identification, especially when comparing mass spectra in large databases.[89] The abundance of identified and quantified metabolites in GC databases, along with the standardisation of the technique, allows for high throughput untargeted analysis of GTE hepatotoxicity to address gaps in the knowledge of biochemical pathways affected by GTE.

1.6.3 Mass spectrometry

Two platforms are used to identify and quantify metabolites: nuclear magnetic resonance (NMR) and mass spectrometry (MS). NMR has the advantage of non-destructive analysis and simple sample preparation.[90] However, due to the relatively low sensitivity of NMR,[90] MS has become the preferred analytical technique due to its high sensitivity and resolution.[88]

A mass spectrometer is an instrument that can ionise molecules from a sample mixture and identify compounds based on their mass-to-charge ratio (m/z). The mass spectrometer has three main components to detect metabolites: the ionisation source, which ionises all fragments that enter; the mass analyser, that separates ions based on their m/z; and the detector, where the fragments strike a conductive surface to amplify the signal. A computer is attached to the mass spectrometer to visualise mass spectra and collate data for all compounds detected.[84-86] (Figure 1.5).

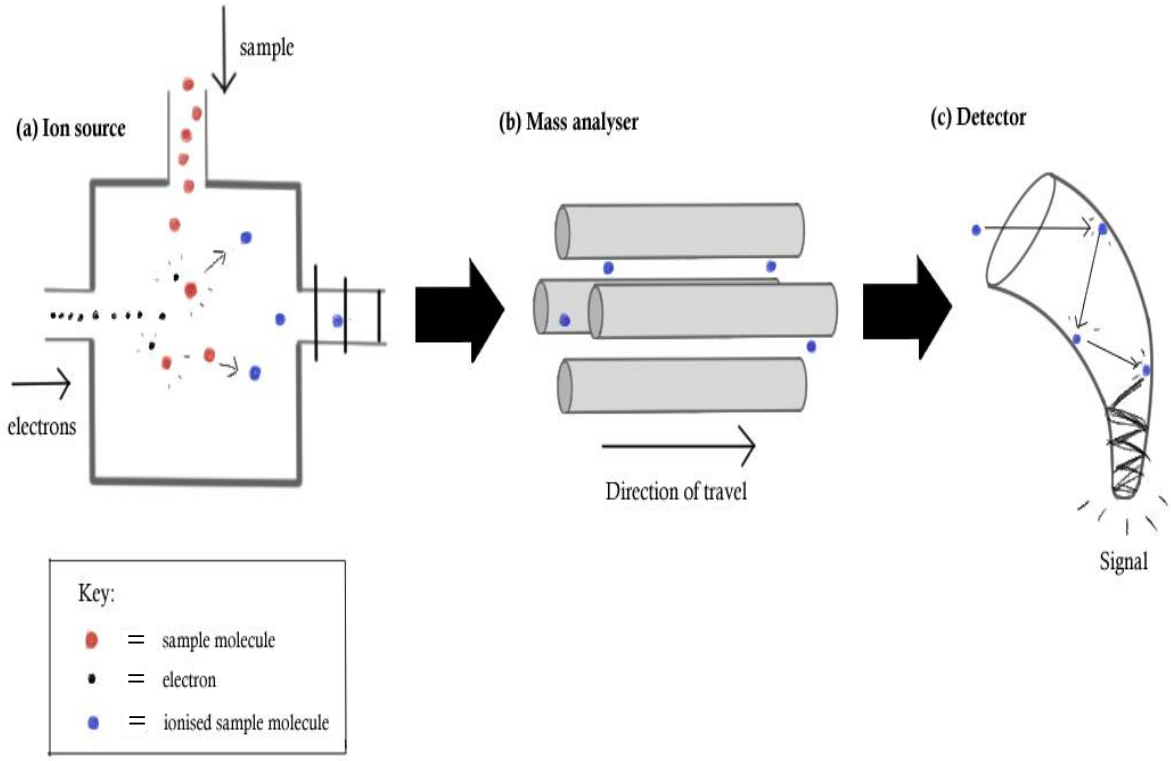

Figure 1.5. Schematic of general MS workflow. (**a**) The sample molecules (red) and electrons (black) collide at a perpendicular angle to ionise the sample molecules (blue). (**b**) The mass analyser separates the ionised sample molecules according to their m/z. An example of a mass analyser is a single quadrupole, as shown above. (**c**) The detector detects the molecule and amplifies the signal by striking a conductive surface to create more ions. A computer collates the data.

1.6.3.1 Electron ionisation

The ionisation source ionises individual compounds to allow them to be manipulated within the electromagnetic field of the mass analyser. In GC-MS, the capillary column attaches to the sample inlet port of the MS ionisation source. The most common ionisation source when MS is coupled with GC is electron ionisation (EI).

EI uses an electron beam to directly bombard the vaporised sample molecules with electrons at 70 electron volts (eV).[86] Known as a 'hard ionisation' technique, it

uses high energy to fragment the ions and forms a unique fragmentation pattern per compound/ion.[86] A heated filament ejects electrons perpendicular to the flight path of the sample molecules within the ionisation chamber. This causes the sample molecules to form positive ions, which move toward a series of lenses using a positively charged repeller and into the mass analyser. Finally, a vacuum draws out any uncharged molecules.[85,86] The fragmentation pattern formed in EI is unique to a molecule and, by comparing the pattern to available mass spectral databases, such as the National Institute of Standards and Technology (NIST), unknown compounds can be identified. EI is particularly useful in the identification of unknown compounds that have not been added to a spectral database as it conveys structural information as well as information about molecular weight.

1.6.3.2 Single quadrupole mass analyser

Popular mass analysers used in MS include single and triple quadrupole (QQQ) analysers. A single quadrupole analyser consists of a set of four cylindrical rods where adjacent rods have a direct current (DC) and radio frequency (RF) signals applied to them.[86] The ions move through the quadrupole at a stable trajectory based on their m/z. At a particular DC/RF combination, only ions of matching m/z will travel across the quadrupole toward the detector whilst keeping the DC/RF ratio constant. All other ions are deflected into the rods until their matching DC/RF combination is applied. The full m/z range is scanned by oscillating the DC/RF signals, usually from low to high voltages.

QQQ operates in the same fashion as single quadrupole, except that three sets of quadrupoles are utilised. The second quadrupole, Q2, only applies an RF signal

instead of both DC and RF. A collision gas is added in Q2 to allow for further fragmentation of ions, and their m/z is measured using the final quadrupole, Q3.[85] Although QQQ has greater selectively and sensitivity,[84] it is more often applied in targeted analysis of compounds due to its potential in identifying unique fragmentation patterns.[77] Quadrupoles, in general, have lower costs, can detect a dynamic range of m/z (100 to 4000), and tolerate higher pressures compared to other mass analysers.[88]

1.6.4 Data processing and deconvolution

The output from MS analysis is called the 'mass spectrum'. A typical mass spectrum shows the relative abundance on the y axis and the m/z on the x axis in the form of spectral peaks. The substantial amount of data acquired in untargeted analysis in MS can often be difficult to interpret manually. To avoid this, the data first undergoes spectral pre-processing to reduce the amount of background noise and low frequency ions, which removes any irrelevant data not associated with metabolomic changes.[91] Deconvolution of spectra helps resolve overlapping peaks of different features (compound fragments), but relies on the use of existing spectral databases.[91] Finally, normalisation of peak areas occurs using an internal standard to correct for variability in peak areas, and to accurately quantify detected metabolites in a sample.[91]

Metabolomics often uses unsupervised and supervised multivariate analyses to identify metabolites with the most significant changes within a large data set. The most common unsupervised analysis is principal component analysis (PCA), which transforms variables into linear uncorrelated combinations, called principal components, to compress and summarise highly dimensional data.[91,92] Principal

component 1 (PC1) has the most variance, whereas PC2 is orthogonal to, i.e. statistically independent of PC1.[92] Different groups that cluster closer together are highly correlated and show less variance, whereas those that are separated are less correlated.[92] Unsupervised analysis is often used as a pre-processing method before undergoing supervised analysis.

The most common supervised analysis in metabolomics is partial least square-discriminant analysis (PLS-DA). It is similar to PCA in that it compresses highly dimensional data, but includes more variables during analysis, therefore describing variance among different groups of samples and creating a more powerful statistical analysis.[77,91] It is highly accurate when larger numbers of variables are included, especially in mass spectrometry.[93] This can aid in identifying significant metabolic patterns that occur in *in vitro* hepatotoxicity research.[77]

1.7 Concluding statements and aims

CAMs have become increasingly popular, and a growing industry in Australia.[6] Despite purported health benefits, their efficacy and safety remains controversial. The potential importance of these safety risks is heightened as the current literature has suggested that the majority of consumers fail to report CAM use to medical practitioners,[16] which is a major concern among the scientific community.[15,18] GTE has been a particular focus for research into hepatotoxicity with numerous cases having surfaced in the last few years.[60-63] Due to its high abundance, EGCG has been the focus of research into the hepatotoxic effect of GTE.[38,51,65] Researchers have suggested that the possible biochemical pathway for hepatotoxicity is due to oxidative stress mechanisms,[38,52] however this remains inconclusive.

This review has identified gaps in the knowledge of GTE hepatotoxicity, most notably regarding its poorly understood biochemical mechanisms. As it represents the final phenotype of an organism, metabolomics will provide more information on the biochemical mechanism and ascertain whether certain metabolites are up- or down-regulated in response to GTE. Gold standard techniques, such as GC-MS, will be the key to clarifying these pathways. Ultimately, the combination of these techniques will contribute to the ongoing debate of safety and efficacy of GTE and other HCAMs. The literature has attributed tea catechins to inducing oxidative stress through ROS production and glutathione (GSH) depletion, and therefore it is hypothesised that GTE hepatotoxicity will identify biochemical pathways related to ROS production, ATP depletion and GSH depletion.

This pilot study has four main aims to address the growing concerns of GTE hepatotoxicity:

1) Identify and characterise, in detail, the biochemical components of 13 GTE products using untargeted single quadrupole GC-MS metabolomic analysis.
2) Characterise CH, EC, EGC and EGCG abundance in 13 different GTE products using untargeted GC-MS analysis and compare to a NIST standard.
3) Expose HepG2 cells to a commercial GTE product and catechin standards for 24 h at two different concentrations, 0.1 and 1 mg/mL, and measure cell viability using an MTT colorimetric assay to indicate a toxicological response.

4) Yield the intracellular metabolites from HepG2 cells and establish biochemical differences from GTE exposure at 0.1 and 1 mg/mL using untargeted single quadrupole gas chromatography-mass spectrometry (GC-MS) analysis and multivariate statistical analyses.

2. Materials and Methods

2.1 Chemicals

The powdered GTE product was purchased from a local health supplement store in Perth, Western Australia. All chemicals and reagents were used in its highest purity available. (+)-catechin hydrate (CH), (-)-epicatechin (EC), (-)-epicatechin gallate (EGC), and (-)-epigallocatechin gallate (EGCG) were all purchased from Sigma-Aldrich (St Louis, MO, USA). Seven other GTE products and six GTE-containing products, including a GTE NIST standard reference material (SRM), were all obtained from a previous study conducted at Murdoch University and had been purchased from local pharmacies, health food stores and online.

For cell culture, Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin and L-glutamine were all purchased from Sigma-Aldrich (St Louis, MO, USA). For metabolomic analysis, methoxyamine hydrochloride, *n*-alkanes (C_{10}, C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32} and C_{36}), D-$^{13}C_6$-sorbitol, pyridine and MSTFA were also purchased from Sigma-Aldrich. LC-MS grade water and methanol were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2 Catechin abundance analysis

2.2.1 Sample preparation

Seven GTE products and six GTE-containing products were analysed with a GTE NIST standard reference material used as a sample reference. Some products required crushing using a pestle and mortar. 10 mg of each GTE sample was

extracted with 5 mL methanol (2 mg/mL). Any undissolved compounds were left to settle to the bottom of the tube to remove any impurities before analysis. Samples were diluted to 100 µg/mL using MeOH-sorbitol mix. Triplicates of each sample were prepared in 1.5 mL microcentrifuge tubes. Samples were dried in a Vacufuge Concentrator Plus and frozen at -80°C until ready for analysis.

The highest purity of CH, EC, EGC and EGCG were all purchased from Sigma-Aldrich (St Louis, MO, USA). The catechins were diluted to 100 ng/µL in 100% methanol and dried in a Vacufuge Concentrator Plus (Eppendorf, Hamburg, Germany) and frozen at -80°C until ready for analysis. A simplified workflow is detailed in Figure 2.1.

2.2.2 Metabolomic analysis

2.2.2.1 Derivatisation

GTE samples and catechin standards were thawed and 20 µL of 20 mg/mL methoxyamine hydrochloride-pyridine mix added. Tubes were placed in a Thermomixer Comfort (Hamburg, Germany) at 30°C and 1200 rpm for 1.5 h. 40 µL of MSTFA added to the tubes, which were put into the Thermomixer for another 30 mins at 37°C and 300 rpm. Tubes were centrifuged at 16.1 x 10^4 rcf for 1 min. The total volume was transferred to GC vials with glass inserts, and 5 µL of *n*-alkane mix (C_{10}, C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32} and C_{36}) added. Vials were left for 1 h at room temperature prior to instrumental analysis.

2.2.2.2 Instrumental analysis

Untargeted metabolomic analysis was conducted using a Shimadzu GC-2010 Plus gas chromatograph with a 10 μL Shimadzu AOC-20i Auto Injector syringe and AutoSampler coupled to a Shimadzu GCMS-QP2010 series single quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The GC inlet was set to splitless mode and the GC-MS interface was set to 300°C. An Agilent FactorFour VF5-5ms fused silica capillary column was used with high-purity helium 5.0 (Coregas, Yennora, Australia) used as a carrier gas for the analyte with a flow rate of 1 mL/min. The initial oven temperature was programmed at 70°C and increased at a rate of 1°C/min for 6 min, which changed to 5.63°C/min thereon to reach a final temperature of 330°C. This gave a total sample run time of approximately 61 mins. The injector was programmed to allow 5 pre-injection and 5 post-injection methanol washes. Exposure samples and control samples were randomised, with QC samples loaded every fifth sample. 1 μL of each sample was injected with the Shimadzu Auto Injector. The vaporised analytes were ionised using electron ionisation (EI) at 70 eV, with the ion source temperature set to 250°C. The scan rate for the MS was set to 10 scans/s and the scan range set to m/z 50-1000.

2.2.2.3 Data analysis

Data obtained from the GC-MS was imported into and deconvoluted using AnalyzerPro® (v5.5.1) and normalised to the $^{13}C_6$-sorbitol internal standard peak areas. An automated library with all identified peaks was created using the GTE NIST SRM, and the targeted component library added to the data matrix. The data matrix was exported into an Excel spreadsheet and manually checked for presence of the catechins. The full data matrix was exported into The

Unscrambler® X v10.3 to perform PCA analysis to show differences between the different GTE samples, excluding outliers.

The base peak and retention time for each catechin was found in each GTE sample and the fold change of each catechin was calculated, using the GTE NIST standard as the baseline catechin level. Separately, metabolites present in < 75% of replicates were excluded and identified by comparing mass spectra and retention times with the in-house library and NIST database.

SPSS (IBM SPSS Statistics 24) was used to conduct a one-way ANOVA with Games-Howell post-hoc testing at a confidence interval of 95%. $P < 0.05$ was considered statistically significant. All data was considered normally distributed after normality testing.

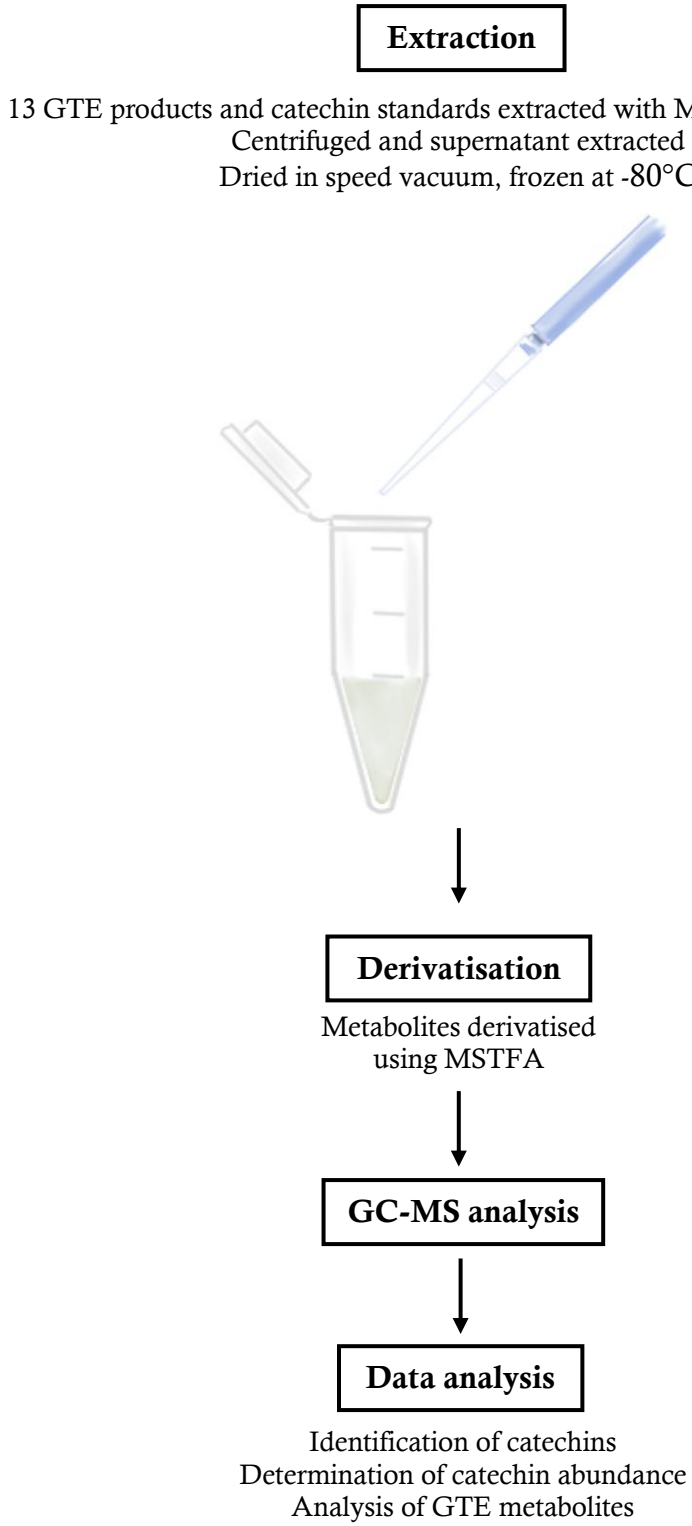

Figure 2.1. Simplified workflow of metabolomic analysis of the 13 GTE products and catechin standards and determination of catechin abundance. Order of events: extraction of GTE products, derivatisation, GC-MS analysis and data analysis.

2.3 GTE cell exposure

2.3.1 Cell culture

A simplified workflow is detailed in Figure 2.3. The human hepatocellular HepG2 cell line was purchased from Sigma-Aldrich (St Louis, MO, USA) sourced from the European Collection of Authenticated Cell Cultures (ECACC acc. no. 85011430), and frozen at -80°C at passage number 31 (P31). Cells were slowly thawed and grown in 75 cm^2 flasks, supplemented with DMEM with 10% v/v FBS, 1% v/v 10,000 U/mL penicillin-streptomycin and 1% v/v 2 mM L-glutamine. Cells were grown in 10 mL of DMEM and incubated at 37°C and 5% carbon dioxide (CO_2) in a Thermo Fisher Scientific Heraeus BB15 Function Line CO_2 incubator (Waltham, MA, USA) at all times. Medium was changed approximately three times per week and cells were passaged once they reached 75-80% confluency.

For passaging and cell counts, cells were trypsinised by removing and discarding 10 mL DMEM from the 75 cm^2 flask and washing with 5 mL pre-warmed sterile PBS. PBS was removed and 2 mL pre-warmed 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) was added and incubated for 2-5 mins at 37°C to dislodge cells from the flask surface. 8mL DMEM was added to terminate trypsination and gently pipetted to break up cell clusters. Cells were passaged 1:3, 1:5 or 1:10 throughout the growth phase for use in exposure experiments or cell counting. For cell counting of 6-well exposure plates, the same procedure was followed using 20% of the original volumes. All experiments were performed in a laminar flow hood in sterile conditions.

2.3.2 Cell counting

Both manual and automatic cell counts were undertaken following exposure experiments. The total cell count (cells/mL) and cell viability (%) were calculated using the Trypan blue exclusion method. Following trypsinisation, a 1:1 v/v ratio of cell mixture and 0.4% Trypan blue stain solution were added to a separate 1.5 mL microcentrifuge tube and gently mixed with a pipette.

For manual counting, 10 µL of the 1:1 v/v ratio was added to each side of a Bright-Line™ Haemocytometer (Hausser Scientific, PA, USA). Viable cells were unstained by the Trypan blue, whereas non-viable cells were blue in colour due to membrane instability. Four 4 x 4 corner squares were used for cell counting, excluding cells falling on the bottom and right border. The total number of cells and viable cells were calculated in each quadrant and the mean was taken. Dilution factors were taken into consideration when calculating the final number of viable cells in cells/mL and recorded.

For automatic counting, 10 µL of the 1:1 v/v ratio was added to each side of a NanoEnTek EVE™ cell counting slide and inserted into a NanoEnTek EVE™ Automatic Cell Counter (NanoEnTek, Seoul, South Korea). Non-viable cells stain dark blue due to the disruption of the cell membrane, whereas the intact cell membrane of viable cells do not take up the dye and appear pale under the microscope. The total cell count, viable cell count, non-viable cell count and cell viability percentage were automatically calculated by the machine and recorded. Both manual and automatic cell counting was conducted in these experiments to determine cell viability and count comparability across all experiments.

2.3.3 Cell treatment

HepG2 cells at passage 31 at a density of 1 x 10^6 cells/mL were seeded into 6 x 6-well plates (2 x quality control (QC), 2 x control and 2 x treatment plates) and DMEM added to make up a final volume of 2 mL per well. Cells were incubated at 37°C and 5% CO_2 and left for 48 h to equilibrate prior to experimentation.

GTE was dissolved at 10 mg/mL in DMEM without any additives (DMEM-/-) and left in the fridge at 2-8°C overnight. The 6 x 6-well plates were taken out of the incubator after 48 h and medium discarded. In the 2 x QC plates, 2 mL of DMEM-/- was added to each well. 1.8 mL of DMEM and 0.2 mL of DMEM-/- were added to 2 x control plates. 1.8 mL of DMEM and 0.2 mL of GTE solution was added to 2 x treatment plates, making the final GTE concentration 1 mg/mL. All plates were incubated for 24 h at 37°C and 5% CO_2. The same procedure was followed for a second round of exposures to make the final concentration in each well 0.1 mg/mL at passage 31. A concentration of 0.1 mg/mL was chosen to reflect the recommended consumer's dose as stated on the packaging. 1 mg/mL was chosen to induce an acute response and to ease identification of metabolites.

2.3.4 Cell viability assay

Cell viability was determined using the thiazolyl blue tetrazolium bromide (MTT) assay. MTT, a yellow-coloured compound, is converted to a dark blue formazan product in the presence of NAD(P)H-dependant oxidoreductases within viable cells to measure cell proliferation (Figure 2.2). The intensity of the blue formazan product is directly proportional to the viability of cells and is measured using colourimetry.

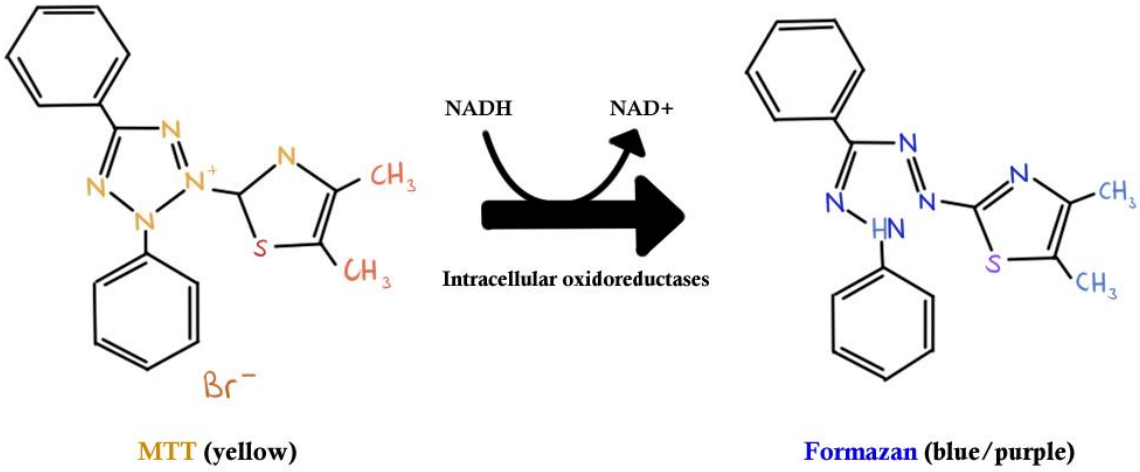

Figure 2.2. Metabolic reaction of MTT (yellow) to formazan (blue/purple). MTT is reduced to formazan in the presence of NAD(P)H and intracellular oxidoreductases. Created using CLIP STUDIO PAINT.

96-well plates (1 x GTE treatment, 1 x CH treatment, 1 x EC treatment, 1 x EGC treatment, and 1 x EGCG treatment) and serum-free DMEM (SF-DMEM) added to a final volume of 100 µL. Cells were left to equilibrate at 37°C and 5% CO_2 and left for 48 h prior to experimentation. Four different concentrations of the catechins (control, 25, 50 and 100 µM) and GTE (control, 0.1, 0.5 and 1 mg/mL) were dissolved in SF-DMEM. Medium was discarded after 48 h and the different concentrations of each treatment added to the wells (Table 2.1). A total of 48 wells per plate (12 wells per treatment) were used for exposures excluding outer wells to reduce any potential edge effect.

Table 2.1. Concentrations of each treatment sample (GTE, CH, EC, EGC and EGCG) and their associated column number for the 96-well plate. The concentration is given as mg/mL for GTE and μM for catechin treatments.

Column number	Treatment concentrations				
	GTE	**CH**	**EC**	**EGC**	**EGCG**
3-4	Control	Control	Control	Control	Control
5-6	0.1	25	25	25	25
7-8	0.5	50	50	50	50
9-10	1.0	100	100	100	100

The CellTiter 96® Non-Radioactive Cell Proliferation Assay protocol (Promega, Madison, WI, USA) was used throughout the cell viability experiment. 15 μL of Dye Solution was added to each well after 24 h and plates further incubated at 37°C and 5% CO_2 for 4 h. 100 μL of Solubilisation Solution was then added to each well and left overnight in a humid environment to allow solubilisation of the formazan product. The absorbance was read at 570 nm wavelength using a Tecan Spark 10M™ multimode microplate reader (Männedorf, Switzerland) with a reference wavelength of 660 nm.

2.4 Metabolomic analysis

2.4.1 Cell harvesting

100 μg/mL of methanol and D-$^{13}C_6$-sorbitol (MeOH-sorbitol) internal standard was prepared to be added to exposure samples. Prior to use, it was kept in a refrigerator at 2-8°C. The mix was pre-chilled in a -80°C freezer for 30 min prior to cell harvesting. The 6 x 6-well plates were placed on ice after 24 h to quench

metabolism. Medium was discarded and cells washed with 500 µL PBS that was discarded. 500 µL of pre-chilled MeOH-sorbitol mix was added to 5 of 6 wells on each plate and cells scraped off using a cell scraper. The cell scraper was washed between each well with 70% ethanol, followed by PBS. Cells were transferred to 1.5 mL microcentrifuge tubes for extraction. The sixth well was left for cell counting, as described in section 2.3.2.

During counting of the sixth well on each plate, the cells exposed to GTE were found to be difficult to lift off of the wells using the appropriate amount of 0.25% trypsin-EDTA and length of incubation. This affected the true viability and total cell count; therefore, the experiment was repeated with the amount of 0.25% trypsin-EDTA and length of incubation adjusted to 500 µL and 8 minutes, respectively, to prevent inaccurate and unreliable cell counts.

2.4.2 Extraction of metabolites

Harvested cells were placed in a Precellys 24™ Tissue Homogeniser at 6500 rpm for 2 x 20 sec cycles (Bertin Technologies, Montigny-le-Bretonneux, France). The cells were removed and placed in a centrifuge (Eppendorf, Hamburg, Germany) for 5 min at 16.1 x 10^4 rcf. Supernatant was removed in equal volumes (approximately 300 µL) and added to fresh 1.5 mL microcentrifuge tubes. Tubes with supernatant were placed in an Eppendorf Concentrator Plus for approximately 2 h and placed in a -80°C freezer once dried. Derivatisation and instrumental analysis were conducted as per sections 2.2.3 and 2.2.4.

2.4.3 Data analysis

Data obtained from the GC-MS was imported into and deconvoluted using AnalyzerPro® v5.5.1 (SpectralWorks, Runcorn, United Kingdom). Data was normalised to the peak area of the $^{13}C_6$-sorbitol internal standard and cell counts. QC samples were loaded onto the program and an automated library was created using identified peaks found in the QC samples. The targeted component library was then added to the GTE exposure sample data matrix. The data matrix was exported into an Excel spreadsheet and metabolites present in < 75% of replicates were excluded.

The data matrix was then exported into The Unscrambler® X v10.3 (CAMO Analytics, Oslo, Norway) to perform PCA and PLS-DA analysis for visual representation of differences between the control and exposure samples. The data matrix was $\log_{10}$ transformed and mean centered prior to PCA analysis using non-iterative partial least squares algorithm, cross validation and no rotation. The resulting PCA plot was used to help discriminate differences between the two sample groups. A supervised PLS-DA analysis was also conducted to identify the most probable metabolites involved in discriminating between the treatment groups. The loadings data matrix was subsequently extracted, and the unknown metabolites were identified by comparing mass spectra and retention times with the in-house library of metabolite standards (Metabolomics Australia, Murdoch University node, Australia) and the National Institute of Standards and Technology (NIST) mass spectral database (v2.3). Metabolite identification was based on the four levels of metabolite identification currently described in the metabolomic literature.[94] The three important outputs given from a mass spectrum

search in the NIST database is the percentage probability, the Match and Reverse Match (R. Match) factors. The percentage probability indicates a compound that has similar or dissimilar mass spectra to other compounds. A higher percentage indicates a compound with dissimilar mass spectra to other compounds, and vice versa.[95] The Match factors have a score out of 999 to denote the most likely mass spectral match between the unknown compound and the library spectrum. An unknown spectra is prone to yielding lower Match factors,[95] and was factored into the chosen criteria.

A metabolite was considered a match if the probability was ≥ 20% and the Match and R. Match factor ≥ 700. If two out of three criteria are met, the compound was putatively identified but there was less confidence in the correct metabolite identified. A metabolite was identified based on its closest compound class if only meeting one of these criteria and its compound class was noted, such as "unidentified amino acid", along with its retention time (RT). Any metabolite not meeting this criteria was denoted at "unknown" and its RT noted. Table 2.2 outlines the criteria followed for metabolite identification.

Table 2.2. The four levels of metabolite classification and their chosen criteria for identification. The criteria were chosen based on the NIST mass spectral database output.

Classification	Criteria
Compound identified	≥ 20% probability ≥ 700 Match ≥ 700 R. Match
Compound putatively identified	2/3 criteria met
Compound class putatively identified	1/3 criteria met
Unknown compound	None of criteria met

SPSS (IBM SPSS Statistics 24) was used to conduct a Student's *t*-test or one-way analysis of variance (ANOVA) with Bonferroni post-hoc testing, as appropriate, with a confidence interval of 95%. $P < 0.05$ was considered statistically significant. All generated data was considered normally distributed after normality testing.

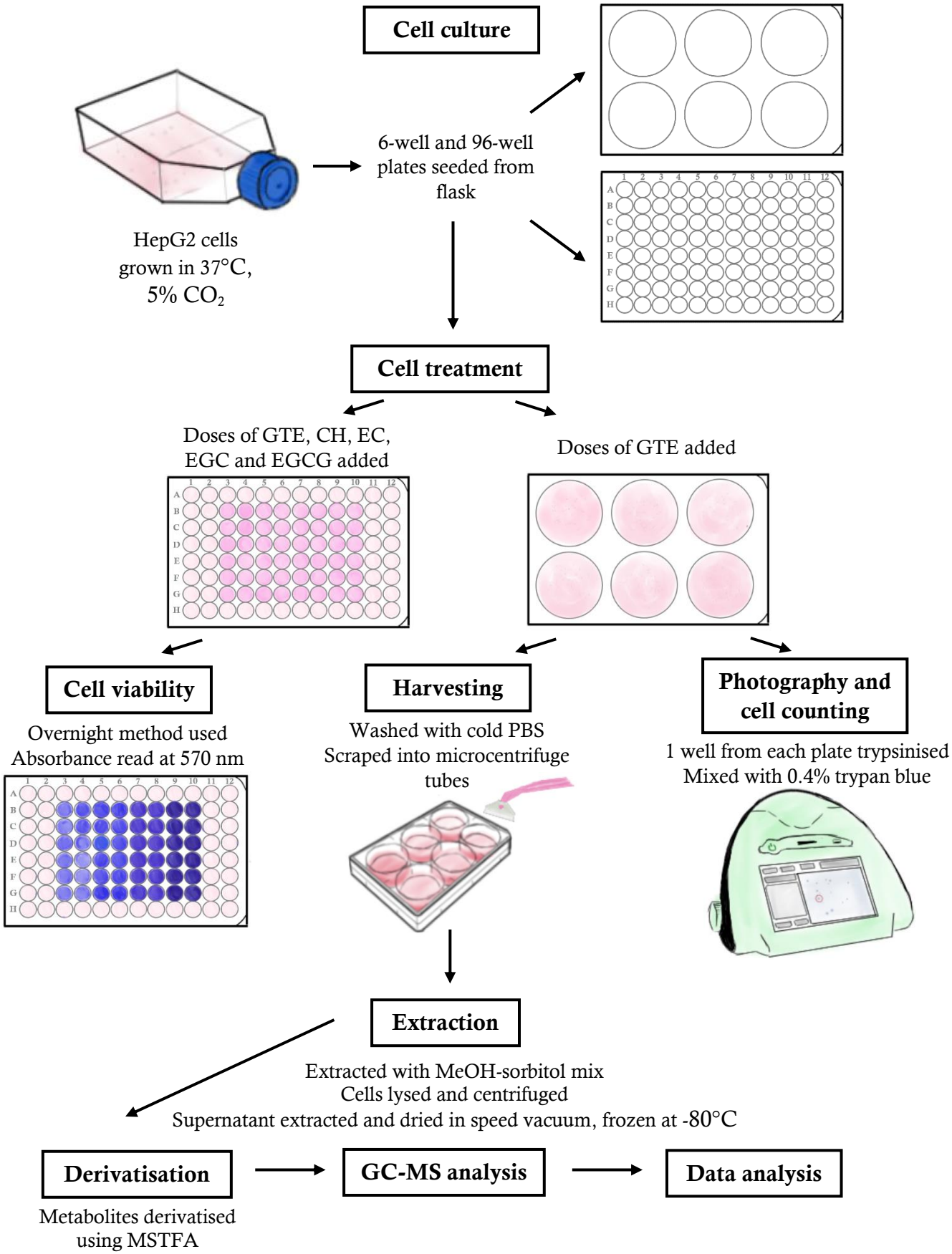

Figure 2.3. Simplified workflow for GTE exposure experimentation, as described in sections 2.2-2.4.5. Order of events: Cell culture, cell treatment, cell viability testing, harvesting, photography and cell counting, extraction, derivatisation, GC-MS analysis and data analysis.

3. Results

3.1 Composition of GTE

3.1.1 Chemical analysis of catechin standards

Standards of CH, EC, EGC and EGCG were analysed using GC-MS. ECG standard could not be obtained within the budget of the project, so was not included. The retention time (RT) and base peak of each catechin standard was determined (Table 3.1). Within a mass spectrum, the base peak is the tallest peak with the greatest relative intensity (abundance), as seen in Figure 3.1. The RT and base peak of CH and EC were almost identical, where the RT was 44.5 and 44.2 min, respectively, and both were observed to have a base peak of m/z 368. Due to the structural similarity of EC and EGC (Figure 1.1), the RTs were very similar (44.2 min and 45.15 min, respectively), differing by 0.95 min. However, the base peak for EC and EGC were m/z 368 and m/z 456, respectively, differing by m/z 88. EGCG had the longest RT of 55.20 mins with a base peak m/z 648 due to the structural dissimilarity between EGCG and the other catechin standards.

Table 3.1. Retention time (mins) and base peak (m/z) results of GC-MS analysis for catechin standards CH, EC, EGC and EGCG.

Catechin	RT (mins)	Base peak (m/z)
EC	44.20	368
CH	44.50	368
EGC	45.15	456
EGCG	55.20	648

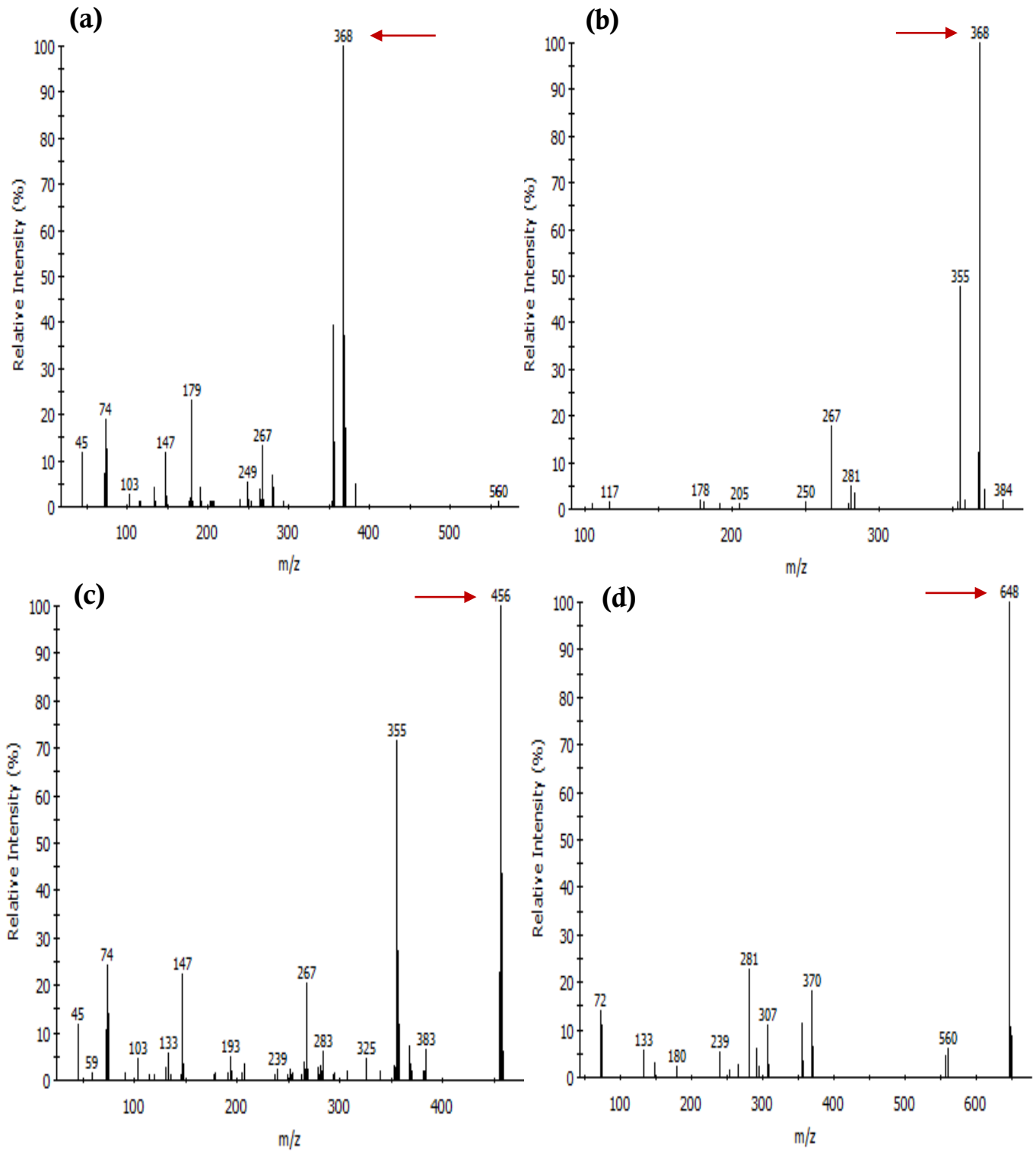

Figure 3.1. GC-MS mass spectra output for (**a**) CH, (**b**) EC, (**c**) EGC and (**d**) EGCG. Each peak shows relative intensity (%) and associated *m/z*. Red arrows indicate the base peak in each catechin standard.

3.1.2 Chemical analysis of GTE samples

Figure 3.2 shows the PCA scores plot comparing the GTE and GTE-containing products. PC-1 explained 16% of the variance and explained the most variance between each GTE product. PC-2 explained 12% of the variance in the data, separating the NIST SRM from the dense cluster of GTE 290, 295, 300, 304 and 305 samples. The grouping of samples along PC-1 and PC-2 showed there was relatively little variation in metabolite data between GTE 290, 295, 300, 304 and 305.

Table 3.2 shows the RT and base peak for the 16 compounds that contributed most to the variance between each GTE product, and the top matches as identified via the NIST mass spectral database. Statistical significance is indicated if there was at least one statistically significant difference between GTE products. In total, 16 metabolites contributed to the most variance between each GTE product. A mixture of carbohydrates, fatty acids, carboxylic acids and organic compounds were present in each product. Many of the top matches had similar base peaks, with five of the 16 metabolites having a base peak of *m/z* 57, three with a base peak *m/z* 147, and two with a base peak of *m/z* of 117. Many of the top matches appeared to be similar despite differing RTs. An example is 9H-Carbazole-1-carboxylic acid, 4-(1H-indol-3-yl)-, methyl ester, which has the same base peak and three different RTs of 45.482, 47.713 and 51.790 mins.

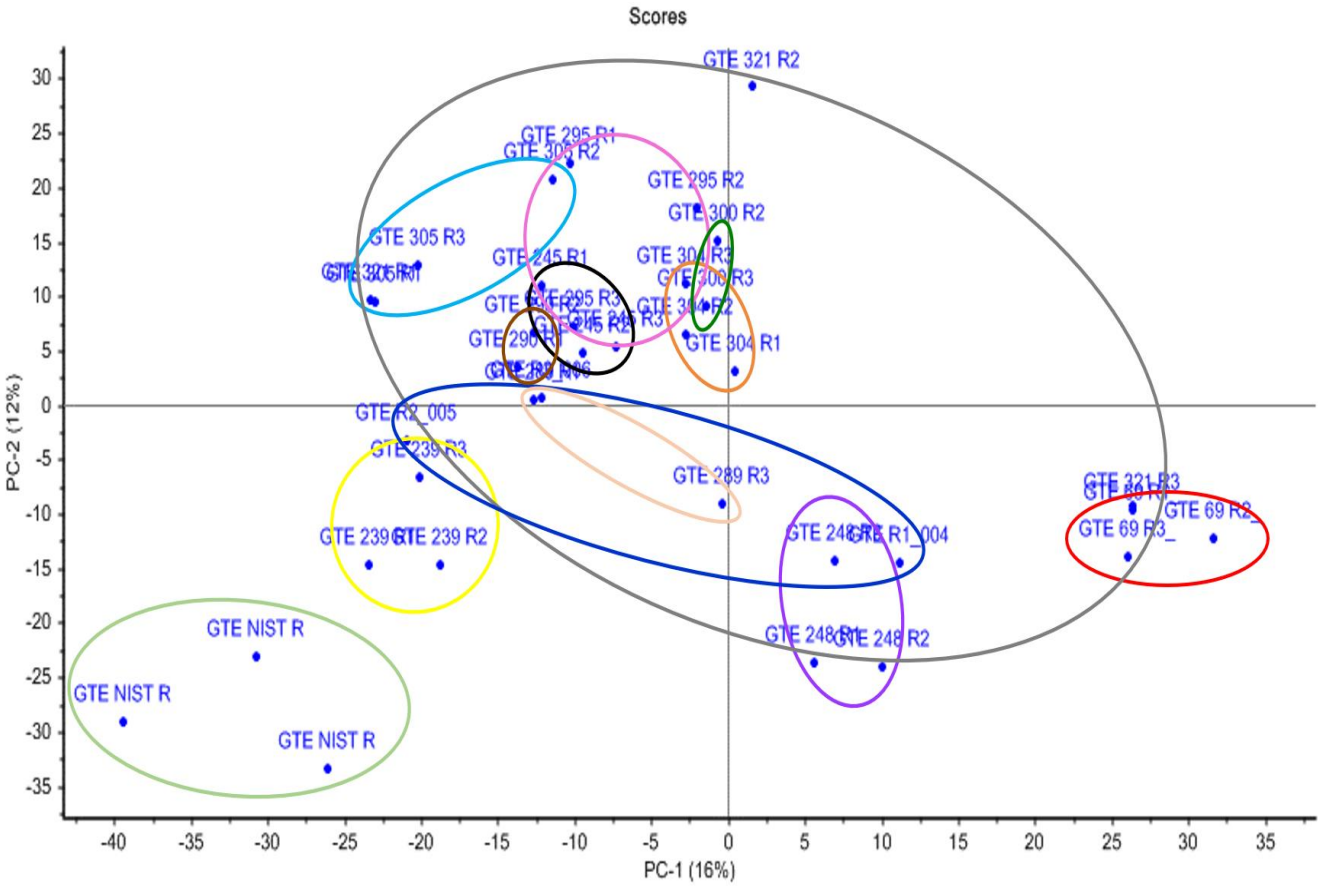

Figure 3.2. Principal component scores of the GC-MS data comparing GTE products. Grouping of sample sets is represented by coloured ovals (GTE NIST SRM = pale green; GTE 69 = red; GTE 239 = yellow; GTE 245 = black; GTE 248 = purple; GTE 289 = pale orange; GTE 290 = brown; GTE 295 = pink; GTE 300 = dark green; GTE 304 = dark orange; GTE 305 = blue; GTE 321 = grey; purchased GTE product = dark blue). Outliers were removed prior to analysis. N = 3 for each sample.

Table 3.2. Retention time (mins), base peak (*m/z*) and the top match identified from the NIST database for compounds contributing most to the variance as identified through PCA. Statistical significance was observed using a one-way ANOVA with Games-Howell post-hoc testing as indicated by * = $p < 0.05$.

RT (mins)	Base peak (*m/z*)	Top match
13.202	147	N-Benzhydrylidene-1-(2,4,6-trimethylphenyl)ethylamine N-oxide*
13.480	113	1-Piperidinecarboxaldehyde*
15.013	228	Ethanol, 2-(methylamino)-, N-trifluoroacetyl, O-tert-butyldimethylsilyl)
16.517	110	N-(tert-Butyldimethylsilyl)trifluoroacetamide*
17.612	107	N-Methyl-N-phenyl-N'-(3-ethoxyphenyl)-urea
18.228	184	N-(tert-Butyldimethylsilyl)-2,2,2-trifluoro-N-methylacetamide
19.792	82	3-Methyl-4-oxo-2-pentenoic acid
30.478	147	Galactose oxime, 6TMS derivative
30.592	147	Benzylmalonic acid, 2TMS derivative*
32.737	117	Palmitic Acid, TMS derivative
35.912	117	Stearic acid, TMS derivative
43.108	57	Timolol methylboronate
45.482	57	9H-Carbazole-1-carboxylic acid, 4-(1H-indol-3-yl)-, methyl ester
47.713	57	9H-Carbazole-1-carboxylic acid, 4-(1H-indol-3-yl)-, methyl ester
49.810	57	4-Ethyl-2-octanol
51.790	57	9H-Carbazole-1-carboxylic acid, 4-(1H-indol-3-yl)-, methyl ester

3.1.3 Relative catechin levels

Twelve GTE and GTE-containing products were analysed compare the catechin abundance to the GTE NIST SRM. GTE products were collected as part of a previous study conducted at Murdoch University, which were purchased from

local health stores, pharmacies, and online. GTE 239, 245, 295, 305, 321, and the GTE product purchased for this study were all labelled as GTE products. GTE 69, 248, 289, 290, 300 and 304 were labelled as products containing GTE within their ingredients. All twelve GTE products and the NIST standard underwent GC-MS metabolomic analysis to observe the relative abundance of each catechin in each product compared to the NIST standard. All statistical analysis was conducted using a one-way ANOVA with Games-Howell post-hoc testing to compare each product to the NIST standard.

Table 3.3-3.5 shows the relative level of each detectable catechin in each tested GTE product. CH was the only catechin that was not detectable in the NIST standard and the GTE products, and therefore removed from further analysis. EC could not be detected in 7 of the 12 products tested (GTE 69, 245, 289, 295, 300, 304 and 321), with 4 of them being GTE-containing products. Of the 5 products in which EC was detected, 4 showed a higher relative amount compared to the GTE NIST standard. Three products (GTE 239, 305 and the GTE product purchased for this study) showed a significant increase of EC of 3.047-fold, 10.311-fold and 9.998-fold, respectively ($p < 0.026$), with GTE 305 having the largest increase ($p < 0.001$). GTE 290 had a 1.138-fold increase in relative EC level, although was not significant ($p > 0.05$). GTE 248 had a 0.442-fold decrease compared to the NIST standard, the only decrease observed, which was not statistically significant ($p > 0.05$).

EGC could be detected in 4 of the 12 products (GTE 69, 289, 295 and 300), of which the majority were GTE-containing products. 7 of the 8 GTE products detected for EGC had a lower relative level, ranging from 0.083 to 0.849-fold decrease in EGC. GTE 245 was determined to have the lowest relative level of

0.083-fold ($p < 0.008$), and GTE 305 was the only product with a higher relative level with a 1.556-fold increase, although this was not statistically significant ($p > 0.05$).

Across the products, EGCG was more varied, with some products having a higher and others a lower relative level compared to the NIST SRM. Similar to EGC, EGCG would not be detected in 4 products (GTE 69, 289, 295 and 300) of which the majority were GTE-containing products. Significantly different EGCG levels were only seen in GTE 239 ($p < 0.040$) and GTE 305 ($p < 0.001$), of which GTE 305 had the highest relative level of 4.602-fold. In terms of lower relative levels, the most notable was GTE 245 which experienced a 0.091-fold decrease, however no statistical significance was observed ($p > 0.05$).

There were only 3 GTE products (GTE 69, 295 and 300) where no catechins were detected, of which two were GTE-containing product and the other a GTE product. 5 of the 12 products had all three catechins detected (GTE 239, 248, 290, 305 and the GTE product purchased for this study). Of particular interest is GTE 305, which had a higher relative level of all catechins compared to the NIST standard, which were statistically significant for two compounds ($p < 0.001$). GTE 248 had the opposite effect, with a lower relative level of all catechins, of which one compound was statistically significant ($p < 0.005$). EC was less reliably detected compared to EGC and EGCG, where three products were detected for the presence of EGC and EGCG only (GTE 245, 304 and 321).

Table 3.3. Relative level of EC for each GTE sample (n= 3) from the GTE NIST SRM. Key: green arrow = fold change above NIST SRM; red arrow = fold change below NIST SRM; **-** = EC not detected. Statistical significance was observed using a one-way ANOVA with Games-Howell post-hoc testing as indicated by * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

	EC
GTE product	**Relative level (fold difference)**
GTE 69	**-**
GTE 239	3.047* ⬆
GTE 245	**-**
GTE 248	0.442 ⬇
GTE 289	**-**
GTE 290	1.138 ⬆
GTE 295	**-**
GTE 300	**-**
GTE 304	**-**
GTE 305	10.311*** ⬆
GTE 321	**-**
Purchased GTE product	9.998* ⬆

Table 3.4. Relative level of EGC for each GTE sample (n= 3) from the GTE NIST SRM. Key: green arrow = fold change above NIST SRM; red arrow = fold change below NIST SRM; **-** = EGC not detected. Statistical significance was observed using a one-way ANOVA with Games-Howell post-hoc testing as indicated by * = $p < 0.05$; ** = $p < 0.01$.

	EGC
GTE product	**Relative level (fold difference)**
GTE 69	**-**
GTE 239	0.849 ↓
GTE 245	0.083** ↓
GTE 248	0.171** ↓
GTE 289	**-**
GTE 290	0.759 ↓
GTE 295	**-**
GTE 300	**-**
GTE 304	0.101** ↓
GTE 305	1.556 ↑
GTE 321	0.193* ↓
Purchased GTE product	0.712 ↓

Table 3.5. Relative level of EGCG for each GTE sample (n= 3) from the GTE NIST SRM. Key: green arrow = fold change above NIST SRM; red arrow = fold change below NIST SRM; **-** = EGCG not detected. Statistical significance was observed using a one-way ANOVA with Games-Howell post-hoc testing as indicated by * = $p < 0.05$; ** = $p < 0.01$.

EGCG

GTE product	Relative level (fold difference)
GTE 69	-
GTE 239	2.246* ⬆
GTE 245	0.091 ⬇
GTE 248	0.623 ⬇
GTE 289	-
GTE 290	1.276 ⬆
GTE 295	-
GTE 300	-
GTE 304	0.133 ⬇
GTE 305	4.602** ⬆
GTE 321	0.247 ⬇
Purchased GTE sample	2.384 ⬆

3.2 Analysis of GTE exposure

Prior to metabolomic analysis, the human hepatocellular HepG2 cell line was cultured and exposed to 0.1 and 1 mg/mL GTE to examine cell growth, morphology and viability compared to untreated cells. Morphology was observed using a light microscope and cells were photographed at 100 x magnification prior to cell count using the Trypan blue exclusion method. Both manual and automated cell count were carried out in order to compare cell count and viability across all experiments conducted.

3.2.1 Cell growth and morphology

Figure 3.3 depicts the morphology of cells when untreated and exposed at 0.1 or 1 mg/mL GTE. HepG2 cells treated with 0.1 mg/mL GTE appeared to have no profound morphological changes compared to the control. Cell density was roughly equal across all treatments, but there were morphological changes after 24 h exposure to 1 mg/mL GTE. There was minor aggregation of cells as the GTE concentration increased compared to the control which affected the overall appearance of the cells. The cells began to lose their slightly elongated shape and became more spherical in 1 mg/mL GTE-treated cells. The most noticeable change was the brown staining of cells exposed to 1 mg/mL GTE, whereas the other two treatments remained unstained.

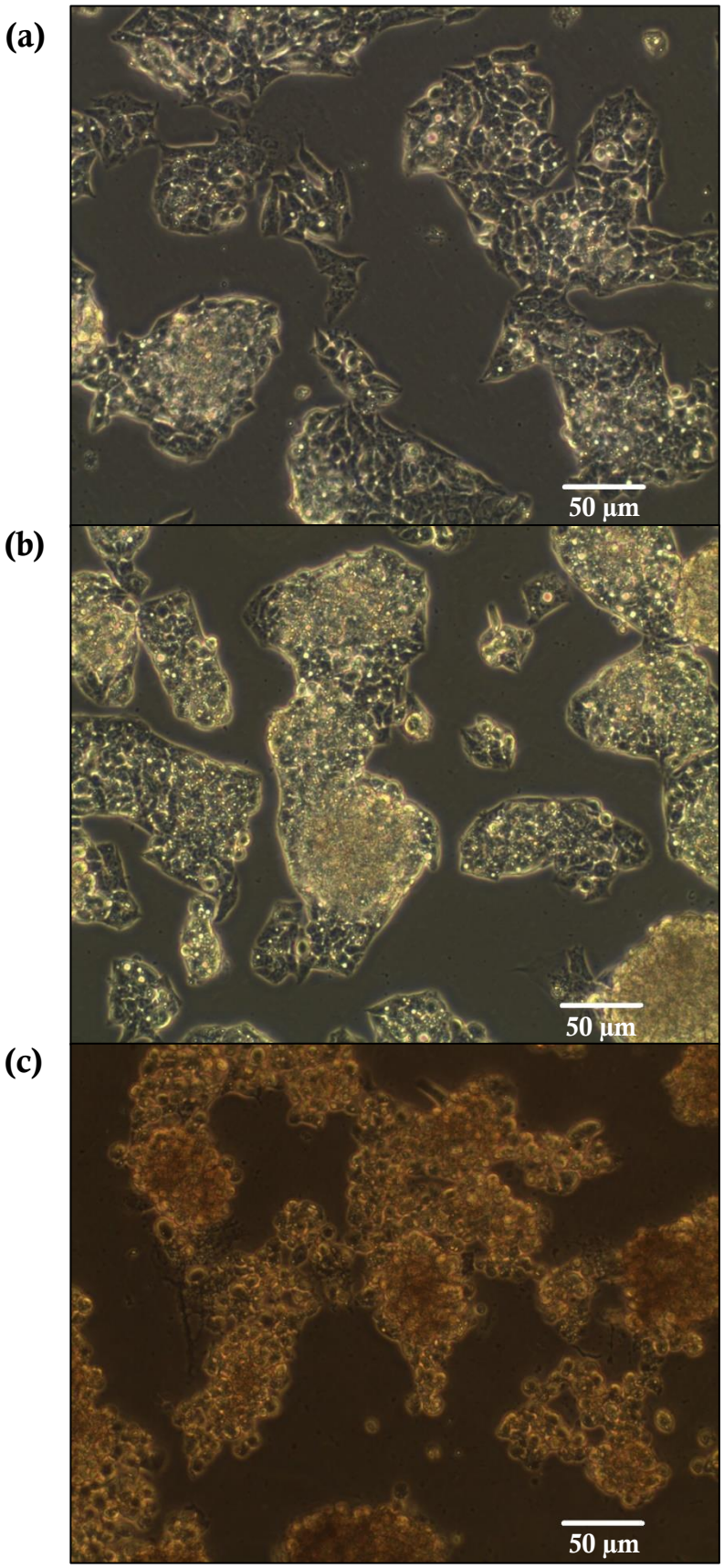

Figure 3.3. Light microscope photographs of (**a**) untreated, (**b**) 0.1 and (**c**) 1 mg/mL GTE-treated HepG2 hepatocellular cells after 24 h at 100 x magnification. Scale bar indicates 50 µm.

3.2.2 Manual vs semi-automated cell counting

Table 3.6 depicts the average total cell count, viability and their associated relative standard deviation (RSD) for the 0.1 mg/mL exposure data set using both manual and semi-automated cell counting. Using the manual cell count method, the average total cell count for the control was 2.62 x 10^6 (± 1.42 x 10^6) cells/mL, a 22% increase from cells treated with 0.1 mg/mL GTE (2.04 x 10^6 ± 2.7 x 10^5 cells/mL). However, the RSD for the total cell count was substantially higher for the control than for 0.1 mg/mL GTE exposure, with RSDs of 54.35% and 13.44% for the control and 0.1 mg/mL data, respectively. The average cell viability for the control was 95% (± 2.83), whereas it decreased by 2.25% to 92.75% (± 4.35) upon exposure to 0.1 mg/mL GTE. Average cell viability showed a considerably lower RSD compared to the average total cell count, with RSDs of 2.98% and 4.69% for the control and 0.1 mg/mL exposure data, respectively.

Average total cell count using the semi-automated method detected fewer cells compared to the manual method, where the average total cell count was found to be 9.38 x 10^5 (± 1.89 x 10^5) cells/mL for the control and 9.7 x 10^5 (± 1.58 x 10^5) cells/mL upon 0.1 mg/mL GTE exposure. In contrast to the manual method, the semi-automated method detected 3.3% greater number of cells in 0.1 mg/mL GTE-exposed cells compared to the control. Statistical significance was observed between the manual and semi-automated methods for the GTE ($p < 0.001$), but not between the controls ($p > 0.05$). This was similar for the average cell viability, where 0.1 mg/mL GTE-exposed cells experienced a 1.25% increase in viability (96.5 ± 3.11% and 97.75 ± 1.50% for the control and 0.1 mg/mL GTE-exposed cells, respectively). Both the control and GTE-treated cells did not show any

significance between the manual and semi-automated methods ($p > 0.05$). The semi-automated method had a considerably lower RSD in both the total cell count and viability as a result.

Table 3.7 shows the average total cell count, viability and RSD between the control and 1 mg/mL GTE-exposed cells using both manual and semi-automated cell counting. For the manual method, the average total cell count was 6.0×10^5 ($\pm 2.8 \times 10^5$) cells/mL and 1.08×10^6 ($\pm 6.7 \times 10^5$) cells/mL for the control and 1 mg/mL GTE-exposed cells, respectively. The RSD in both treatments was substantially higher with RSDs of 47.31% and 62.49%, respectively. This was slightly lowered using the semi-automated method, with the RSDs being 44.03% and 38.85% for the control and 1 mg/mL GTE-exposed cells, respectively. Significant differences were observed in control and GTE-treated cells between automatic and manual cell counting ($p < 0.020$ and $p < 0.001$, respectively). Cell viability was much lower in the manual method compared to the semi-automated method, with a difference of 18.08% for the control and 29.34% for GTE-exposed cells. This was reflected in the RSD, which decreased by 12.92% and 24.61% in control and GTE-exposed cells, respectively. Significant differences were observed in control and GTE-treated cells between automatic and manual cell counting for viability ($p < 0.001$).

Upon comparison between manual and the semi-automated methods at both treated concentrated, the RSDs were generally higher and therefore showed less accuracy in the manual method. The semi-automated cell count data was more reproducible and was therefore reported for subsequent data.

Table 3.6. Average total cell count, cell viability and associated RSD for both manual and semi-automated cell count techniques for 0.1 mg/mL exposure data. Total cell count and cell viability data is represented as the mean ± standard deviation, and RSD represented as percentage. N = 4 for each treatment.

MANUAL				
	Total cell count (10^6 cells/mL)	**RSD (%)**	**Cell viability (%)**	**RSD (%)**
Control	2.62 ± 1.42	54.35	95.00 ± 2.83	2.98
0.1 mg/mL	2.04 ± 0.27	13.44	92.75 ± 4.35	4.69
SEMI-AUTOMATED				
	Total cell count (10^5 cells/mL)	**RSD (%)**	**Cell viability (%)**	**RSD (%)**
Control	9.38 ± 1.89	20.13	96.50 ± 3.11	3.22
0.1 mg/mL	9.70 ± 1.58	16.28	97.75 ± 1.50	1.53

Table 3.7. Average total cell count, cell viability and associated RSD for both manual and semi-automated cell count techniques for 1 mg/mL exposure data. Total cell count and cell viability data is represented as the mean ± standard deviation, and RSD represented as percentage. N = 12 for each treatment.

MANUAL				
	Total cell count (10^6 cells/mL)	**RSD (%)**	**Cell viability (%)**	**RSD (%)**
Control	0.60 ± 0.28	47.31	68.75 ± 14.61	21.25
1 mg/mL	1.08 ± 0.67	62.49	49.58 ± 17.85	36.00
SEMI-AUTOMATED				
	Total cell count (10^5 cells/mL)	**RSD (%)**	**Cell viability (%)**	**RSD (%)**
Control	3.64 ± 1.60	44.03	86.83 ± 7.23	8.33
1 mg/mL	2.63 ± 1.02	38.85	78.92 ± 8.99	11.39

3.2.3 Cell counts and cell viability

Figure 3.4 depicts the mean cell viability for untreated and GTE-treated cells as determined by the Trypan blue exclusion method. Viability of cells treated with 0.1 mg/mL GTE increased by 1.30% (101.30 ± 1.50%) after 24 h exposure compared to untreated cells (100 ± 3.11%), which was not statistically significant ($p > 0.05$). Figure 3.4b shows that the viability of cells treated with 1 mg/mL GTE decreased by 9.11% (90.88 ± 9.00%) compared to untreated cells (100 ± 7.23%), a statistically significant difference as determined by Student's *t*-test ($p < 0.027$).

Figure 3.5 shows the mean total cell count (cells/mL). As per Figure 3.5a, the mean total cell count for untreated cells and cells treated with 0.1 mg/mL GTE was 9.38×10^5 ($\pm 1.89 \times 10^5$) and 9.70×10^5 ($\pm 1.58 \times 10^5$) cells/mL, respectively. With only a difference of 3.20×10^4 cells/mL (3.35%), there was no observable difference, nor was it statistically significant ($p > 0.05$). Figure 3.5b shows the mean total cell count decreasing from 3.64×10^5 cells/mL ($\pm 1.60 \times 10^5$) to 2.63×10^5 cells/mL ($\pm 1.02 \times 10^5$) when treated with 1 mg/mL GTE, a difference of 1.01×10^5 cells/mL (27.7%). Although visually different, Student's *t*-test confirmed that there was no statistical significance between the means ($p > 0.05$).

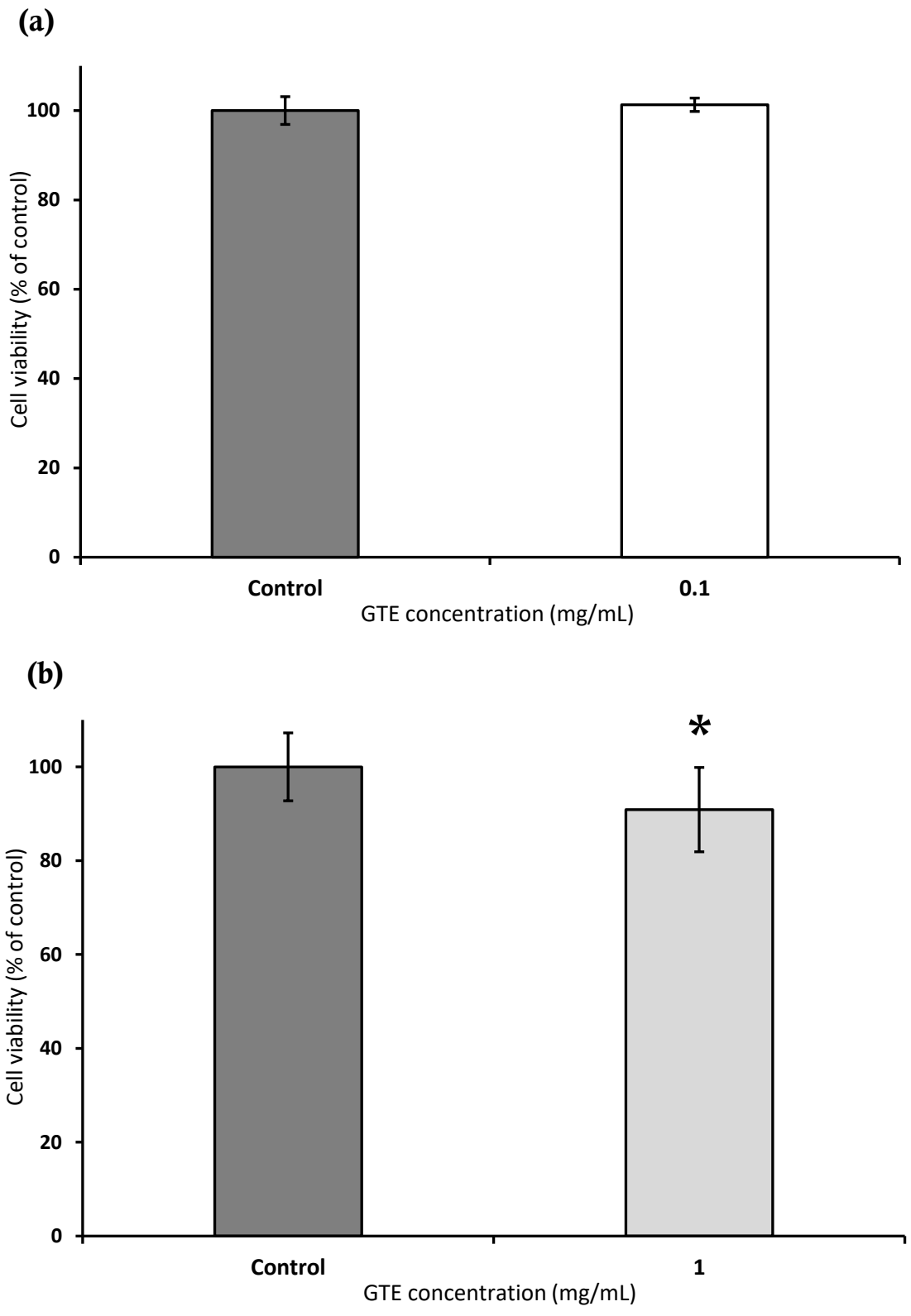

Figure 3.4. Mean cell viability (%) for untreated and GTE-treated HepG2 cells after 24 h exposure to (**a**) 0.1 (n = 4) and (**b**) 1 mg/mL GTE (n = 12), as determined by Trypan blue exclusion. All values are expressed as the percentage of the control ± standard deviation. * indicates significant difference ($p < 0.05$) between untreated and GTE-treated cells.

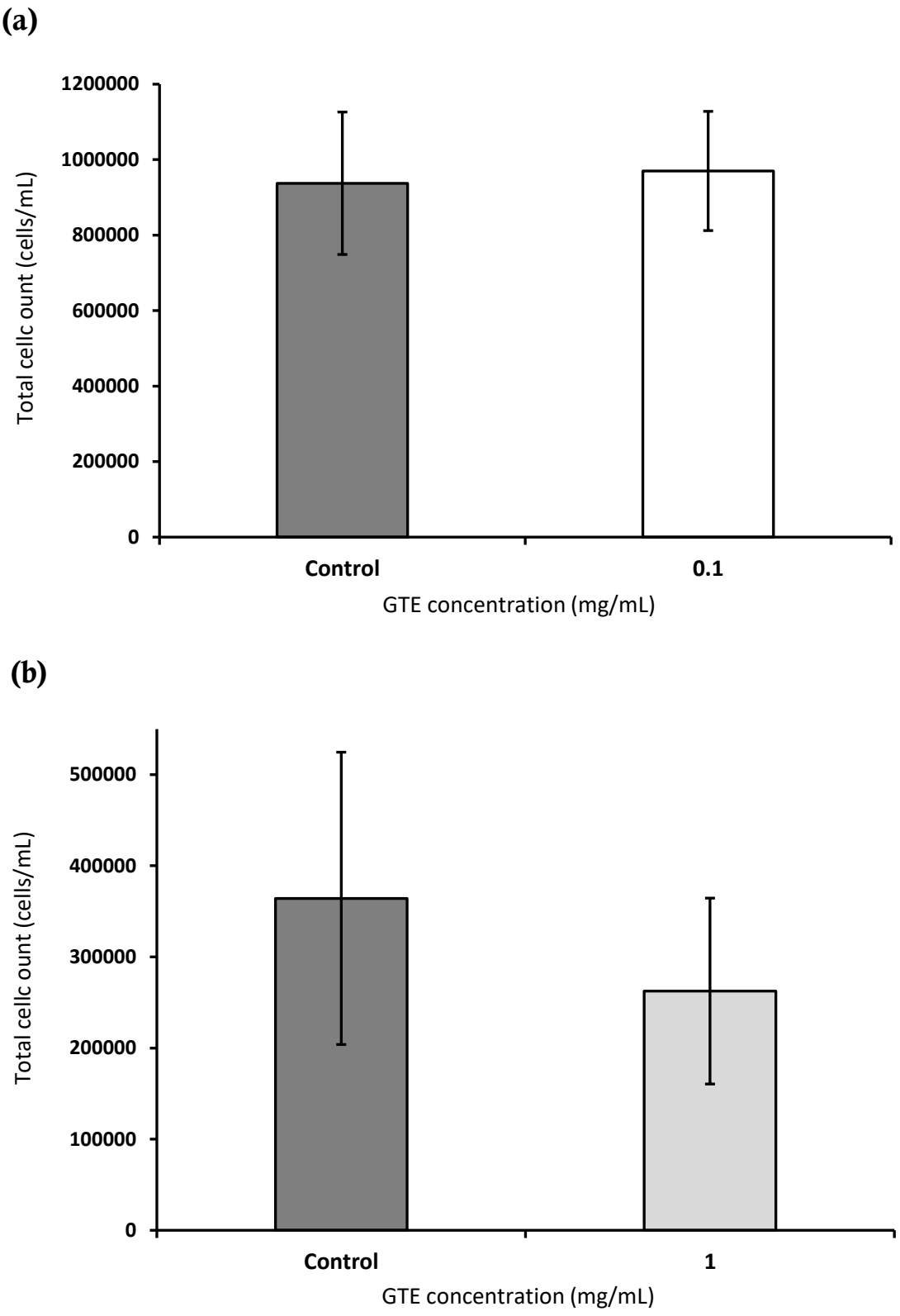

Figure 3.5. Mean total cell count (cells/mL) for untreated and GTE-treated HepG2 cells after 24 h exposure to (**a**) 0.1 (n = 4) and (**b**) 1 mg/mL GTE (n = 12), as determined by Trypan blue exclusion. All bars are represented as the mean ± standard deviation.

3.2.4 MTT assay

To determine the effect of CH, EC, EGC, EGCG and GTE concentration on cell viability, human hepatocellular HepG2 cells were cultured in 96-well plates at a range of concentrations for GTE (0.1, 0.5 and 1 mg/mL) and catechin standards (25, 50 and 100 μM) and cell viability measured using an MTT assay. Viable cells use its intracellular NAD(P)H-dependent oxidoreductases to convert MTT (yellow) to formazan (blue/purple) as seen in Figure 2.2. The colour intensity was measured at 570 nm and is directly proportional to cell viability. Statistical analysis was conducted using a one-way analysis of variance (ANOVA) with Bonferroni post-hoc testing to compare each concentration to the control.

Figure 3.6 shows the mean cell viability of untreated, CH, EC, EGC, EGCG and GTE-treated HepG2 cells after 24 h exposure. In general, the viability increased above the control with the exception of cells exposed to 50 and 100 μM EGC.

EGCG treatment at all concentrations showed a linear increase in cell viability as concentration increased. Cell viability increased by 9.57% (± 7.26; $p < 0.001$), 14.04% (± 5.36; $p < 0.001$) and 17.50% (± 6.35; $p < 0.013$) compared to the control when treated with 25, 50 and 100 μM, respectively. Similar to EGCG, EC-treated cells also followed a positive linear relationship between cell viability and EC concentration. Compared to untreated cells, the cell viability increased by 1.86% (± 1.64), 3.77% (± 2.92) and 4.99% (± 2.05) when cells were exposed to 25, 50 and 100 μM, respectively. Although the difference in cell viability was less pronounced than EGCG treatment, there were significant differences between the control and 50 ($p < 0.004$) and 100 μM ($p < 0.001$) EC treatments. Treatment at 25 μM EC was not significant compared to the control ($p > 0.05$).

Cells treated with EGC followed a different trend compared to EGCG and EC. 25 μM EGC treatment had a very similar cell viability (0.12% ± 3.87) compared to the control, but the cell viability reduced to 94.28% (± 4.71) at 50 μM, a significant decrease of 5.72% ($p < 0.015$). The cell viability increased to 97.99% (± 6.75) at 100 μM, with no statistical significance observed ($p > 0.05$). Cells treated with various concentrations of CH had similar cell viability and varied by 0.91-2.52% compared to the control. The cell viability increased to 100.91% ± 0.039 at 25 μM CH from the control and reached its highest cell viability at 50 μM (102.52% ± 2.17). However, a one-way ANOVA determined there was no significance between the 50 μM CH and the control ($p > 0.05$). The cell viability decreased to 101.73% ± 3.59 at 100 μM CH. Overall, CH did not have any statistically significant changes when compared to the control.

Cells treated with various concentrations of GTE demonstrated significant changes compared to the control. Cell viability increased from 100% ± 5.23 to 120.42% ± 6.30 after exposure to 0.1 mg/mL GTE ($p < 0.001$). The cell viability plateaued after exposure to 0.5 ($p < 0.001$) and 1 mg/mL GTE ($p < 0.001$) and remained at a similar cell viability of 123.08% (± 4.76) and 121.58% (± 13.24), respectively.

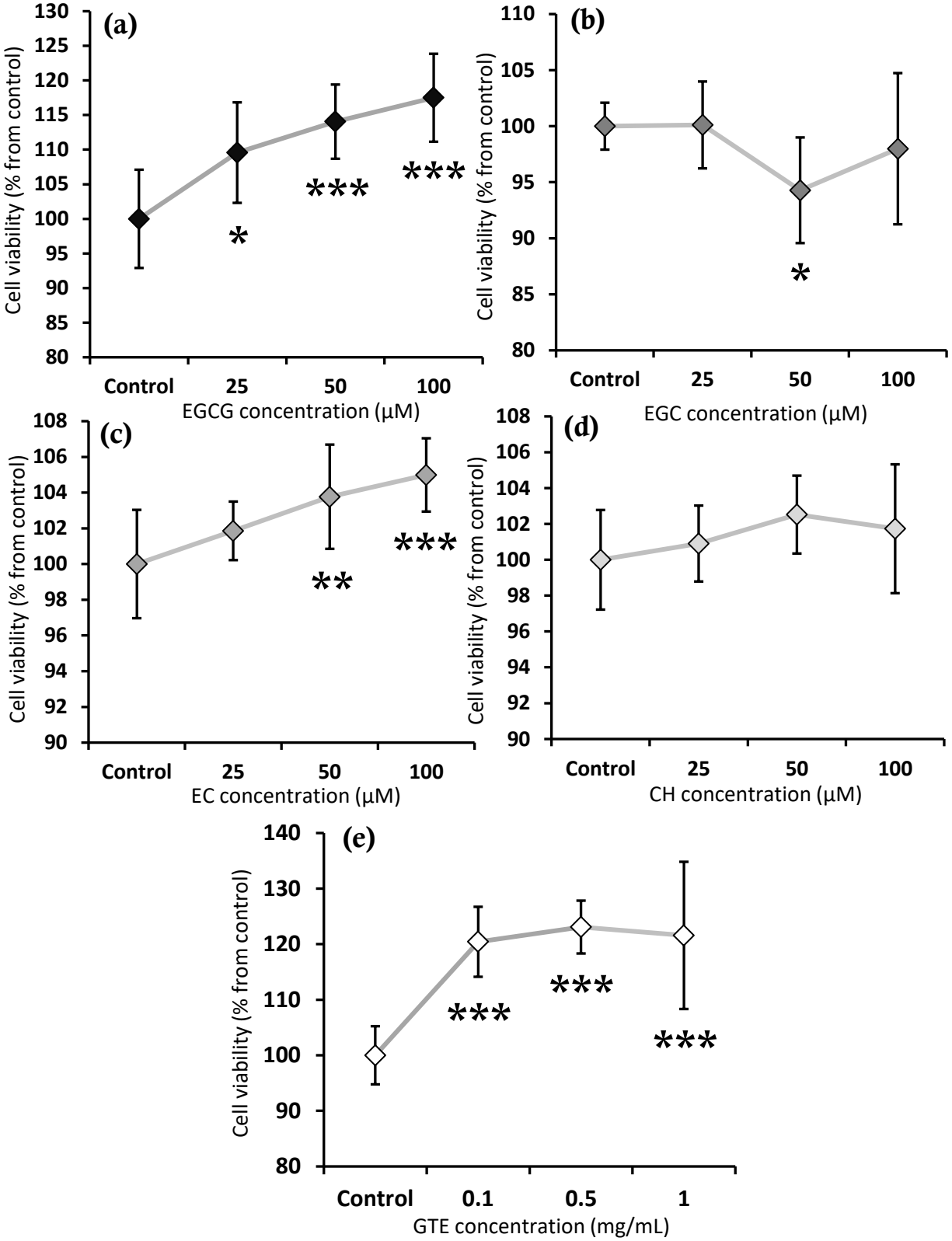

Figure 3.6. Cell viability (%) of (**a**) EGCG, (**b**) EGC, (**c**) EC, (**d**) CH and (**e**) GTE-treated HepG2 cells at different concentrations (n =12) after 24 h exposure. Data is expressed as the percentage from the control ± standard deviation. Statistical significance was tested using a one-way ANOVA with Bonferroni post-hoc testing as indicated by * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

3.2.5 Metabolomic analysis

To analyse biochemical changes that occur in GTE-treated HepG2 cells, untargeted metabolomic analysis was conducted using GC-MS. Differences in intracellular metabolites were analysed at two different GTE concentrations of 0.1 and 1 mg/mL with ten replicates of control and GTE-treated cells after 24 h exposure.

Intracellular metabolites were extracted from the cells and analysed through GC-MS to indicate whether the levels of different metabolites changed with GTE exposure and, consequently, if the same biochemical pathways were affected at different doses. All data was normalised to the internal standard (D-$^{13}C_6$-sorbitol) and cell count. As previously mentioned, difficulties with cell counts for the 1 mg/mL exposures prevented data being normalised to the cell counts. All data was $\log_{10}$-transformed and mean-centered prior to PCA and PLS-DA analysis. The metabolites were identified through comparing mass spectra and retention times to the in-house library and NIST database (v2.3). Metabolite classification was based on the criteria outlined in Table 2.2.

3.2.5.1 1 mg/mL exposure

Figure 3.7 shows the PCA scores plot for intracellular metabolites found in ≥ 75% of replicates for cells exposed to 1 mg/mL GTE for 24 h. GTE-treated cells (red) are clearly separated from the untreated cells (blue) and loosely clustered along PC-1. PC-1 explained 19% of the variance and was the major principal component (PC) that explained variance between GTE-treated and untreated cells. Although the data points are more dispersed compared to PC-1, PC-2 explained 14% of the

variance between untreated and GTE-treated cells. Figure 3.8b shows the loadings plot for each metabolite and was referred to for identification of each metabolite.

Figure 3.8a shows the PLS-DA scores plot. Factor-1 explained 19% of the variance of the x variables (the independent variable, i.e. the metabolomic data) identified as the metabolite peak areas from GC-MS analysis. Factor-1 also explained 86% of the variance of the y variable (dependent variable, i.e. the treatment groups), identified as the two sample sets (untreated and GTE-treated cells). It can be assumed that Factor-1 represented the most variance due to the influence of GTE on the metabolome. In contrast, 7% of the variance was explained in both the x and y variables for Factor-2, and therefore it can be assumed that it experienced equal variance in both variables. After PCA and PLS-DA analysis, the loadings for each data point were exported and metabolites identified via the in-house library and the NIST mass spectral database to investigate the fold change from the control.

Table 3.8 indicates the fold changes of the normalised peak area for each metabolite identified as contributing most to the variance identified from PLS-DA analysis. All metabolites were listed according to their compound class (amino acid, carboxylic acid, oxoacid, carbohydrate, nucleic acid, fatty acid and 'other'). Some metabolites were excluded due to duplication or a compound identified to be a part of the column, making the total number of important metabolites 27. 14 of the 27 metabolites were able to be fully identified ($\geq$ 20% probability, Match and R.Match $\geq$ 700) with a further five metabolites putatively identified. 25 of the 27 metabolites (93%) were decreased and two (7%) were increased in GTE-treated cells compared to the control cells. The data showed an overall decrease in all classes of compounds, most significantly in amino acids, carboxylic acids and

oxoacids. Unidentified sugar_32.270 had the largest increase of 2.224-fold ($p < 0.002$) and unknown_28.850 having the largest decrease of 0.063-fold ($p < 0.001$). The majority of the metabolites appear to be amino acids, which were decreased in all instances. Almost all of the identified amino acids had a significant decrease in the mean peak area ($p < 0.001$), with the exception of L-cysteine ($p > 0.05$). All identified carboxylic acids, 1,2-benzenediol ($p < 0.004$) and unidentified carboxylic acid_22.627 ($p < 0.001$), had a fold decrease compared to the control and showed significant difference between the means. The oxoacids also had a fold decrease and were significant ($p < 0.001$). Although a fold decrease occurred, the nucleic acids and fatty acids did not have a significant difference ($p > 0.05$). 4 of the 9 metabolites that could not be classified were significantly different, with fluoranthene and unknown_28.850 being the most significant ($p < 0.001$), followed by unidentified amine_16.358 ($p < 0.004$) and propanenitrile ($p < 0.025$).

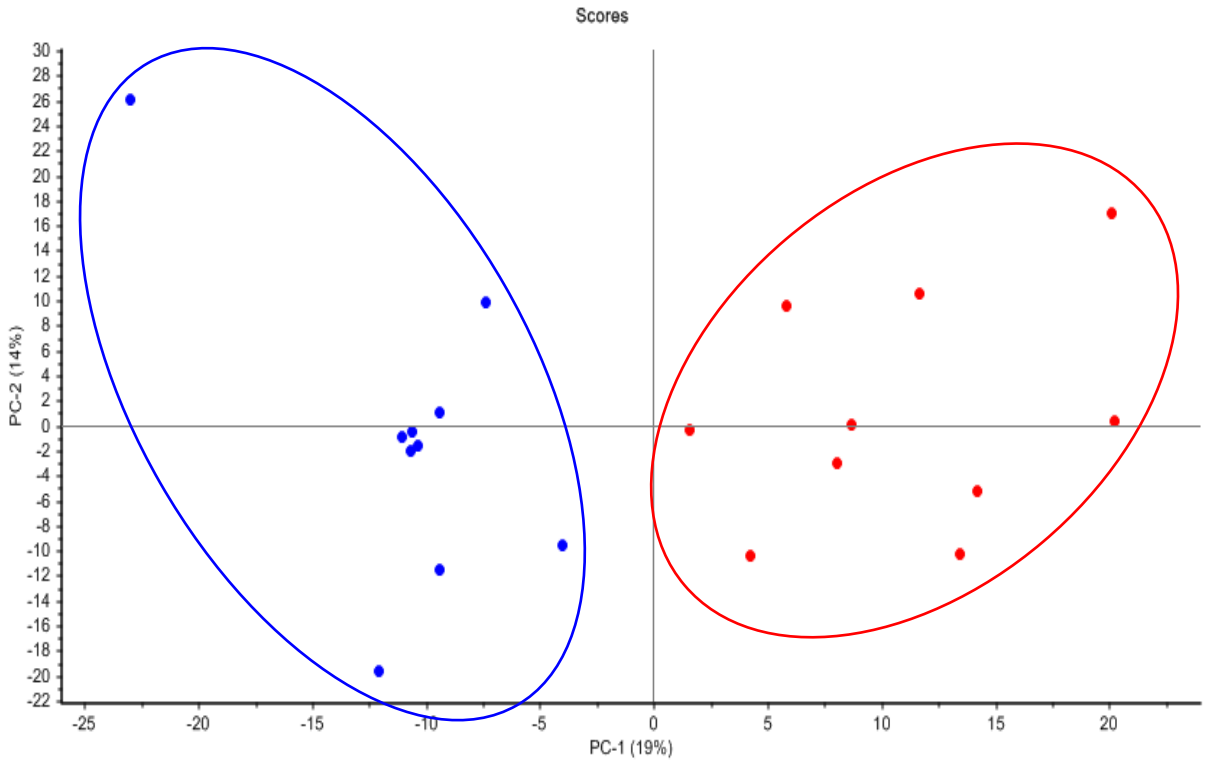

Figure 3.7. Principal component analysis scores plot representing the metabolites contributing most to the variance after 24 h exposure to 1 mg/mL GTE. Grouping of sample sets is represented by coloured ovals (blue = control; red = GTE-treated). N = 10 for each treatment group.

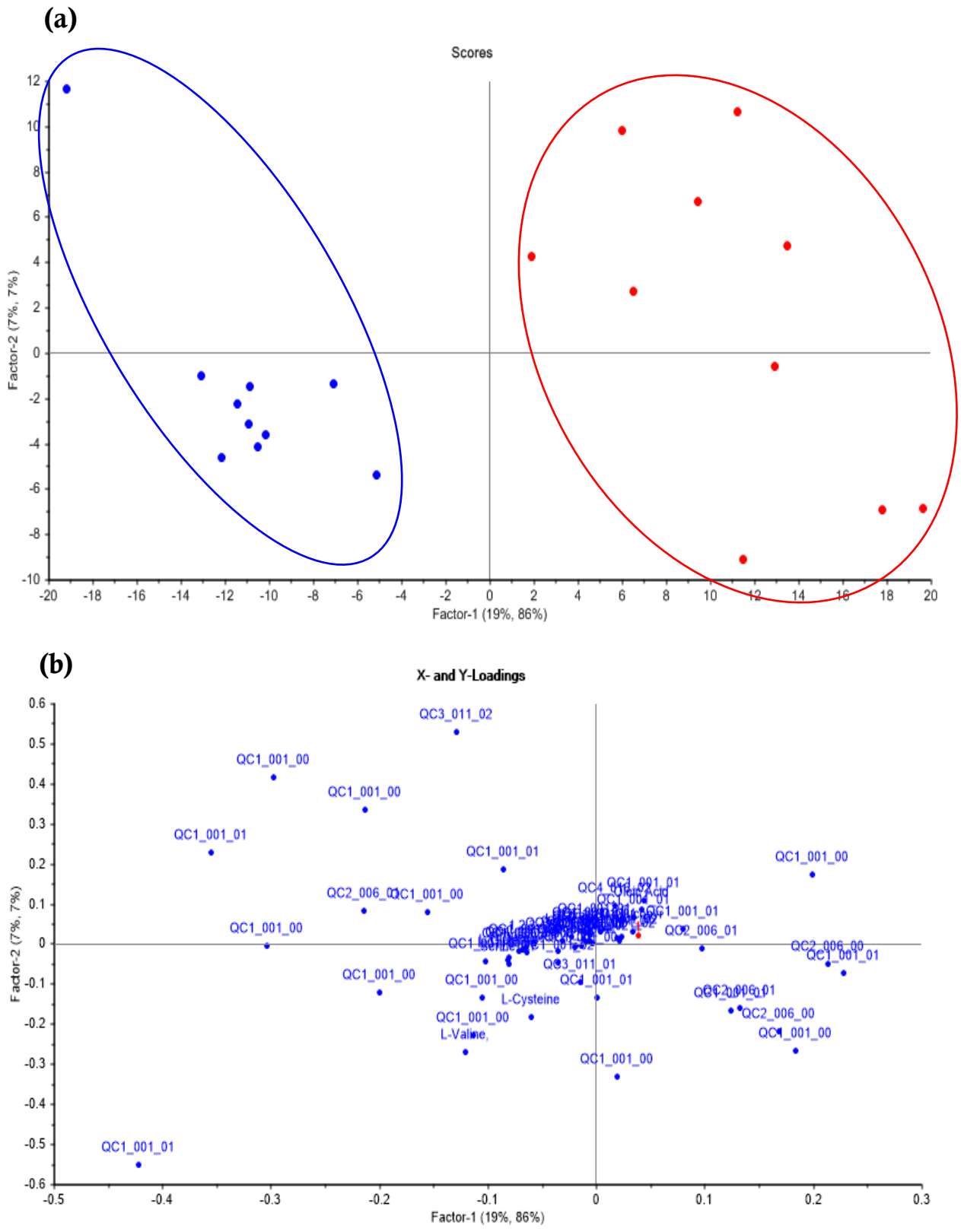

Figure 3.8. Partial least squares-discriminant analysis scores (**a**) and loadings plots (**b**) representing the metabolites contributing most to the variance after 24 h exposure to 1 mg/mL GTE. Grouping of sample sets is represented by coloured ovals (blue = control; red = GTE-treated). N = 10 for each treatment group.

Table 3.8. Metabolites identified by PLS-DA contributing most to the variance from the control and their corresponding fold change upon exposure to 1 mg/mL GTE. Red arrows represent fold decrease observed between GTE-treated (n = 10) and control (n = 10) cells, and green arrows represent a fold increase. Statistical significance was observed using Student's *t*-test as indicated by * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. † indicates putatively identified metabolites.

Identified metabolite	Fold change
Amino acids	
L-Cysteine	0.858 ↓
L-Tyrosine	0.181*** ↓
L-Glutamic acid	0.135*** ↓
L-Threonine	0.134*** ↓
L-Glutamine	0.086*** ↓
L-Isoleucine	0.278*** ↓
L-Valine	0.187** ↓
Phenylalanine	0.195*** ↓
Serine	0.141*** ↓
L-Norvaline†	0.198*** ↓
L-Lysine†	0.238*** ↓
Carboxylic acids	
1,2-Benzenediol†	0.835** ↓
Unidentified carboxylic acid_22.627	0.072*** ↓
Oxoacids	
Phosphoric acid	0.205*** ↓
Citric acid	0.207*** ↓
Carbohydrates	
Unidentified sugar_32.270	2.224** ↑
Nucleic acids	
Uracil	0.497 ↓
Fatty acids	
Unidentified fatty acid_32.377	0.765 ↓
Other metabolites	
Trimethylamine compd. with borane (1:1)	0.447 ↓
Fluoranthene	0.238*** ↓

Nonadecane†	0.936	⬇
Propanenitrile†	0.211*	⬇
Unidentified alcohol_45.467	1.142	⬆
Unidentified amine_16.358	0.191**	⬇
Unknown_28.850	0.063***	⬇
Unknown_37.865	0.836	⬇
Unknown_40.565	0.799	⬇

3.2.5.2 0.1 mg/mL exposure

Figure 3.9 shows the PCA scores plot for untreated and 0.1 mg/mL GTE-treated cells following 24 h exposure. The cluster of GTE-treated cells (red) are more tightly clustered than the untreated cells (blue). PC-1 explained 18% of the variance experienced between untreated and GTE-treated cells, while PC-2 explained 13% of the variance. Figure 3.10a shows the PLS-DA scores plot. Factor-1 explained 10% of the variance in the x variables. It also explained 60% of the variance in the y variables, and therefore can be assumed that Factor-1 represented the most variance due to the influence of GTE on the metabolome. For Factor-2, the x and y variables explained 11% and 16% of the variance, respectively. Figure 3.10b shows the loadings plot for each metabolite and was referred to for identification of each metabolite.

Table 3.9 shows the fold changes of the normalised peak area for each identified metabolite contributing most to the variance identified from PLS-DA analysis when exposed to 0.1 mg/mL GTE. One control sample was removed from analysis due to derivatisation or injection failure. The total number of detected metabolites was 21, of which half were increased. In total, 5 metabolites could be fully identified according to the classification criteria (Table 2.2), with a further 7

putatively identified. Two metabolites, unidentified fatty acid_32.275 ($p < 0.031$) and unknown_26.695 ($p < 0.009$), had the most substantial difference compared to the control. Other metabolites had a smaller fold changes and were not statistically significant.

The majority of metabolites appeared to be amino acids which experienced a mixture of fold increase and decrease but had no significant difference compared to the control ($p > 0.05$). This contrasts with 1 mg/mL exposure, where almost all amino acids experienced a significant fold decrease compared to the control. There were also considerable differences in the amino acids identified. Compared to 1 mg/mL GTE exposure, where 11 different amino acids were identified, only 3 amino acids were identified with 0.1 mg/mL GTE exposure (and 3 unidentified). One amino acid, L-isoleucine, was similar among the different treatment concentrations. 3 carbohydrates and 4 fatty acids were detected in 0.1 mg/mL exposure compared to 1 mg/mL exposure, but no significant differences were found. Neither carboxylic acids, oxoacids nor nucleic acids were detected in this sample set compared to 1 mg/mL exposure.

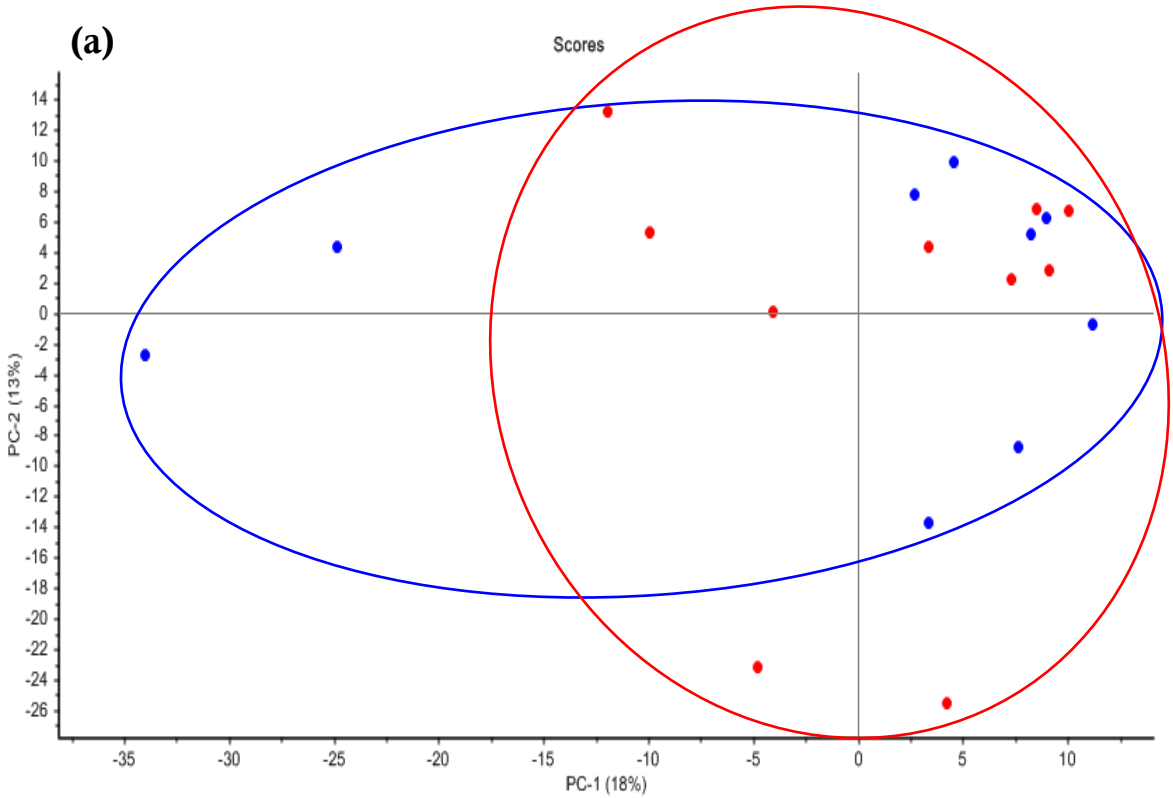

Figure 3.9. Principal component analysis scores plots representing the metabolites contributing most to the variance after 24 h exposure to 0.1 mg/mL GTE. Grouping of sample sets is represented by coloured ovals (blue = control; red = GTE-treated). N = 9 for control and n = 10 for GTE-treated cells.

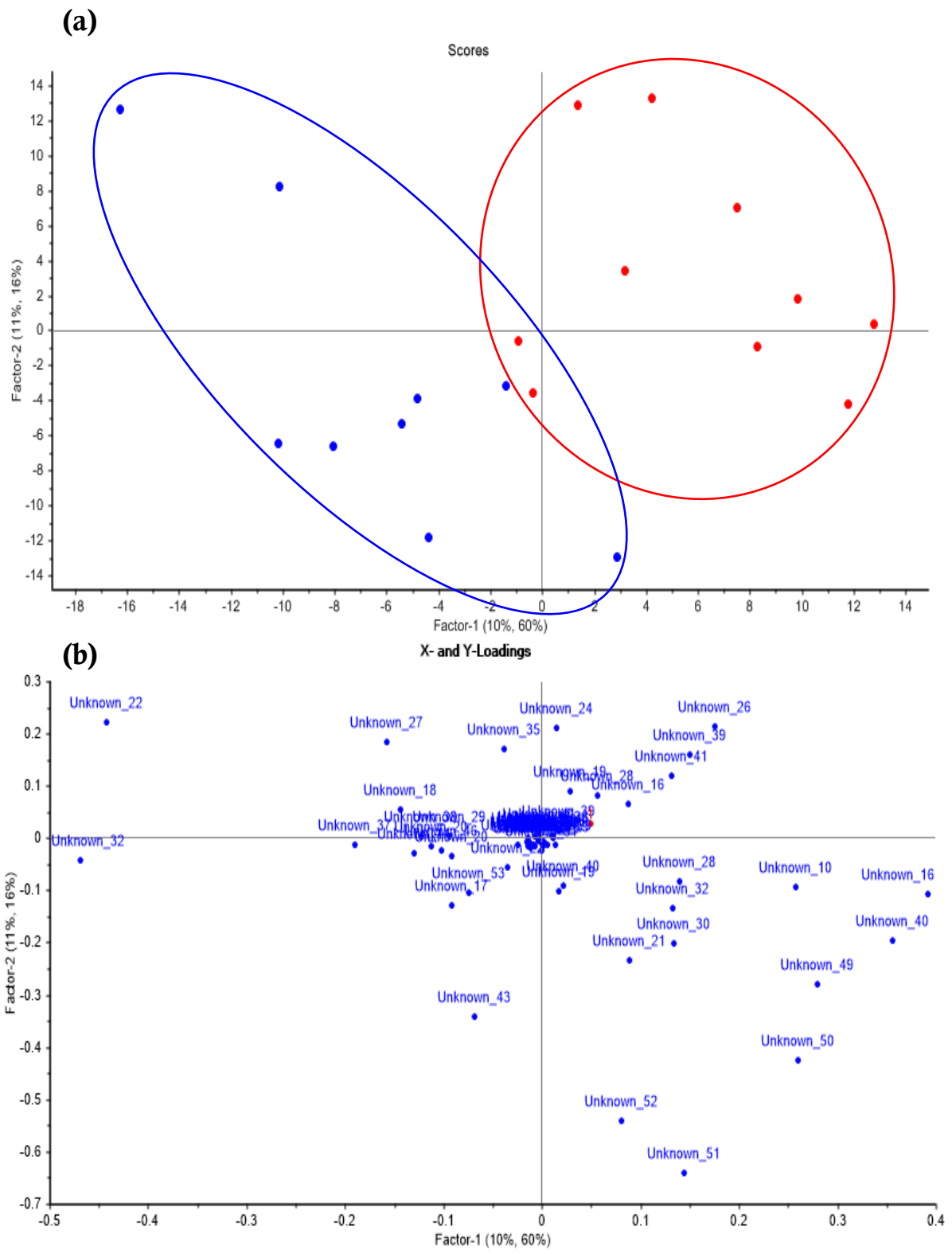

Figure 3.10. Partial least squares-discriminant analysis scores (**a**) and loadings plots (**b**) representing the metabolites contributing most to the variance after 24 h exposure to 0.1 mg/mL GTE. Grouping of sample sets is represented by coloured ovals (blue = control; red = GTE-treated). N = 9 for control and n = 10 for GTE-treated cells.

Table 3.9. Metabolites identified by PLS-DA as contributing most to the variance from the control and their corresponding fold change upon exposure to 0.1 mg/mL GTE. Red arrows represent a fold decrease observed between GTE-treated (n = 10) and control (n = 9) cells, and green arrows represent a fold increase. Statistical significance was observed using Student's *t*-test as indicated by * = $p < 0.05$; ** = $p < 0.01$. † indicates putatively identified metabolites.

Identified metabolite	Fold change
Amino acids	
L-Methionine	1.067 ↑
L-Isoleucine	0.884 ↓
Alanine†	0.957 ↓
Unidentified amino acid_20.437	0.924 ↓
Unidentified amino acid_28.018	1.409 ↑
Unidentified amino acid_28.548	1.161 ↑
Fatty acids	
Decanoic acid	1.166 ↑
Unidentified fatty acid_29.260	0.946 ↓
Unidentified fatty acid_32.275	0.942* ↓
Carbohydrates	
Unidentified sugar_32.257	1.021 ↑
Unidentified sugar_39.198	1.090 ↑
Unidentified sugar_40.038	0.848 ↓
Organic compounds	
2-Phenyl-1,3-oxazol-2-ine†	0.394 ↓
1-ethenyl-2-[(E)-hex-1-enyl]cyclopropane†	0.804 ↓
Unidentified organic compound_27.405	0.980 ↓
Other metabolites	
trans-Butenedioic acid	1.091 ↑
5-Isopropyl-8-methyl-5,6,7,8-tetrahydro-2,4(1H,3H)-quinazolinedione	1.265 ↑
Nonane†	0.974 ↓
DL-Lactamide†	1.069 ↑
Bis(2-(Dimethylamino)ethyl) ether†	1.037 ↑
Unknown_26.695	1.612** ↑

4. Discussion

4.1 Analysis of GTE composition

4.1.1 GTE metabolite analysis

Green tea extract (GTE) is one of the most popular beverages in the world, and is extracted and concentrated for its use as a herbal supplement. Metabolomic analysis was conducted on a variety of GTE and GTE-containing products to understand the biochemical similarities between these products and how differences could impact liver health. The packaging of the product used in this study includes an advisory and warning label indicating the demographic for consumption and the origin of the ingredients. An AUST R or AUST L number is absent from the packaging, indicating that this particular GTE product is not listed in the ARTG and is likely to be regulated as a food product instead of a herbal medicine. All food products are regulated in accordance to the Australia New Zealand Food Standards Code, also known as the Food Standards Code, by their respective state or territory.[96] The Food Standards Code states that a product advertised as a formulated supplementary sports food requires a nutrition information panel on the packaging.[96] An initial concern was the lack of macromolecule nutritional information on the packaging, as studies have detected the presence of sugars and amino acids.[97] However, the small concentrations detected in GTE may be the reason for the lack of nutritional information on the packaging. Therefore, according to the Food Standards Code, the product seems to comply with standard requirements for trade as a food product.

Two fatty acids, palmitic and stearic acid, were detected in each GTE sample. Unsaturated fatty acids are commonly found in plant extracts and can be oxidised to volatile compounds that have characteristic aromas during the manufacturing process, such as 1-penten-3-ol and *n*-nonanal.[97] Alcohols are volatile compounds that contribute to green, floral, lemon, fresh aromas in green tea.[98] The putatively identified compounds, 3-methyl-4-oxo-2-pentenoic acid, ethanol-2-(methylamino), N-trifluoracetyl, O-tert-butyldimethylsilyl and 4-ethyl-2-octanol may be chemical derivatives of such volatile compounds. The presence of a sugar within GTE is also corroborated by other studies.[97,99] The presence of free sugars are highly correlated with inducing the formation of aromatic alcohols in green tea.[97] Galactose oxime, the chemical derivative of galactose, was putatively identified within the GTE samples and other studies have identified glucose, fructose and sucrose in GTE.[99,100]

It is possible that the fatty acids and sugars may contribute to hepatoprotective effects of GTE. Fatty acids are involved in ATP production through their catabolism by β-oxidation to form acetyl CoA, an intermediate within the TCA cycle. It has recently been suggested that supplementation with GTE induces peroxisome proliferator-activated receptor (PPAR) genes involved in the up-regulation of β-oxidation in the liver.[101] The presence of fatty acids in GTE may contribute to increased β-oxidation for hepatoprotective effects, such as fatty liver disease associated with a high fat diet.[101]

Other studies have identified an array of amino acids in GTE,[99,100] which were not identified in this study. This is possibly due to the high replicate percentage that was chosen for this study as many of the GTE metabolites detected were chemicals

used in the derivatisation process. However, it may also be due to the extraction process used in this study. Changes in the extraction method, time of extraction and extraction temperature for the manufacturing of GTE have been shown to vary the amino acid concentration.[102] Conventional methods of tea extraction use heated distilled water, whereas this study used methanol. Using methanol has the advantage of extracting more polar metabolites, but a two-phase system (such as chloroform and water) may be a more efficient method for detecting metabolites that were not extracted.[103]

1-piperidinecarboxyaldehyde is a piperidine, a hydrogenised pyridine, and present in some plants[82] and is known to induce toxicity at acute doses.[104] It is likely that this metabolite is the result of the chemical derivatisation of pyridine before GC-MS analysis. However, it has also been found to be an organic leachable, compounds that are able to migrate from packaging and into the product and are found in trace amounts. As the compound is highly soluble and is frequently observed as an extractable, it increases the risk of harm to an individual.[105] The frequency with which it was observed in these samples suggests that, if it is not derived from the sample preparation process, it may be commonly in GTE products. As the metabolite is known to induce acute toxicity,[104] further research should focus on deducing the toxicity of this compound at chronic doses within GTE products.

Many of the detected metabolites either have no particular function within GTE or were derivatives of chemicals used to derivatise metabolites. This study used MSTFA to derivatise metabolites. Although a popular silylation reagent in plant studies, MSTFA has been shown to increase the number of peaks detected in GC-

MS analysis.[103] The high replicate percentage (≥ 75%) chosen for this study may have affected the true number of metabolites detected in this sample set. Confidence in the identification of most of these metabolites is low, and therefore the aim of identifying and characterising the biochemical components of commercial GTE products was partially fulfilled. Identifying these will need further elucidation to understand the pathophysiology of GTE hepatotoxicity. Limitations in metabolite identification are further discussed in section 4.3.

4.1.2 Variation in relative catechin levels of GTE products

Following on from previous studies into catechin-related hepatotoxicity from GTE,[38,51,65] analysis of the relative catechin levels was conducted between different GTE and GTE-containing products. Catechins were not detected in GTE 69, 289, 295 or 300, with three of these samples being GTE-containing products. GTE 305 consistently had the highest relative levels of EC, EGC and EGCG, with the most significant being a 10.31-fold higher level of EC compared to the NIST SRM. In contrast, GTE 248 had the lowest relative levels, with the most significant being a 0.17-fold lower level of EGC. The GTE sample purchased for this study had a variety of relative levels, with the most significant being a 9.99-fold higher level in EC compared to the NIST SRM.

The most interesting result was from the 10-fold higher level of EC in both GTE 305 and the GTE sample purchased for this study, both of which were significant. EC has been suggested to have the least toxic effect on hepatocytes.[38] Galati et al. suggested that the LD_{50} for rat hepatocytes was over 10,000 μM,[38] the equivalent of approximately 3 mg/mL. As EC comprises only 0.8% of the green tea composition (approximately 0.008 mg/mL in a 1 mg/mL green tea sample),[68] EC

would require a higher concentration to induce a hepatotoxic response. The 10-fold higher level of EC for GTE 305 and the purchased sample would probably not be enough to induce a hepatotoxic response from EC alone, but the variation in levels between samples is concerning.

Relative levels of EGC were generally lower in all products, except GTE 305. The most significantly lower relative levels were in GTE 245, 248, 304 and 321, ranging from 0.083 to 0.193-fold lower. The LD_{50} for EGC has been suggested to be 3000 μM,[38] equivalent to approximately 0.9 mg/mL. As EGC comprises approximately 6% of the total composition in green tea (approximately 0.057 mg/mL in a 1 mg/mL sample),[68] it is not likely that these GTE samples would induce a hepatotoxic response from EGC alone, similar to EC. The relatively low levels of EC and EGC in the purchased GTE product suggests that these catechins may not be responsible for inducing hepatotoxicity in HepG2 cells.

EGCG has been at the centre of hepatotoxicity studies for some time and has been regarded as the most toxic of all catechins.[38,51,65] *In vitro* studies using primary rat hepatocytes have shown hepatotoxicity at a variety of concentrations at seeding densities similar to those used in this study, ranging from 10 - 200 μM (0.005 - 0.09 mg/mL).[38,65] The composition of EGCG is approximately 5% of the brewed amount of green tea,[68] and is much more concentrated in GTE, comprising approximately 17% of the composition.[52] Therefore, it is likely that the GTE samples that showed the highest relative levels of EGCG, GTE 239, 305 and the purchased sample for this study, may induce a more acute hepatotoxic response compared to those with a lower relative level.

The expected proportions of each catechin are only estimates extracted from the literature. As the use of untargeted metabolomics is only a semi-quantitative technique,[106] it prevents the quantification of each catechin from the GTE samples. The data did show that there was considerable variation among catechins levels, suggesting that environmental factors and extraction techniques can change the composition of GTE and its detected metabolites.[47,48,103] The potential for GTE-induced hepatotoxicity for the purchased product used for this study is considerable, and the data brings into question the safety regulations that are associated with the product.

4.2 Analysis of GTE exposure

4.2.1 Cell growth and morphology

Growth and morphological changes in HepG2 cells were observed under a light microscope. Cells appeared to become spherical and brown staining upon exposure to 1 mg/mL GTE. Aggregation was not noted as a significant change in morphology as HepG2 cells are a proliferative cell line and therefore grow in clustered 3-dimensional (3D) structures on a monolayer. There are no current studies with insight on morphological changes in GTE-treated HepG2 cells, but other products high in polyphenols presented similar observations of round shaping of HepG2 cells, such as exposure to grape pomace extract.[107] Jimenez-Lopez and Cederbaum showed that HepG2 cells overexpressing CYP2E1 treated with arachidonic acid and iron experienced rounding of cells, and showed EGCG resolved this phenomenon.[108] The rounding of cells, blebbing (protrusion of cell membrane) and DNA fragmentation are hallmarks of apoptosis.[109] Therefore, it is suggested that GTE may have pro-apoptotic effects on HepG2 cells, and that

transmission electron microscopy (TEM) may be a beneficial tool to further observe the presence of apoptosis in GTE hepatotoxicity, as demonstrated in a similar study.[110]

The most interesting change in morphology was the brown staining of the cells, which was also observed at a macroscopic level. The polyphenol subsets, theaflavins and thearubigins, are a product of oxidised catechins catalysed by endogenous oxidase enzymes during the fermentation of green to black tea to produce the characteristic reddish-brown pigment found in black tea.[43,111] However, these endogenous oxidases are heat-inactivated during production of green tea and therefore cannot formed the pigmented molecules.[111] Another subset of polyphenols, the tannins, may be a plausible cause for the staining of cells. Tannins are soluble molecules that range from yellow to brown pigments and have the ability to complex with multiple macromolecules such as proteins, starch and cellulose.[112] Tannins are separated into hydrolysable and condensed tannins (proanthocyanidins), of which proanthocyanidins play defensive roles against pathogens in many vascular plants.[113] Therefore, it may be possible that the proanthocyanidins dissolved within the medium and complexed with the phospholipids and proteins on the cell membrane of the HepG2 cells to cause reddish-brown staining. Along with observing changes in nuclear morphology, TEM would have confirmed the presence of tannins interacting with the cell membrane, as previously used in a similar study.[113]

As this project was conducted using a hepatocellular carcinoma cell line, there were dissimilar morphological variations observed in liver biopsies in case studies of GTE hepatotoxicity. Many case studies reported evidence of ballooning

degeneration, individual cell necrosis, lobular collapse and disorganised architecture.[60-63] This study found a change in cell architecture to be the only similarities in an *in vivo* setting. It is known that the extrapolation from *in vitro* hepatotoxicity to *in vivo* models is a challenge among researchers. New technologies, such as 3D scaffolds or sheets, may improve extrapolation by emulating the lobule structure of normal liver tissue.[114,115]

4.2.2 Differences in manual and semi-automated cell counting

This study compared the use of a microscope haemocytometer slide and a semi-automated cell counter on the reproducibility on total cell count and viability data using the Trypan blue exclusion method. This was determined by calculating the percentage RSD, which determines the precision of the data set. The RSD was generally higher in the manual method compared to the semi-automated method, indicating the data was varied and less precise.

Cell viability and concentration within cell lines is paramount in the reproducibility of subculturing.[116] Manual cell counting with a haemocytometer has been the most widely used technique, and whilst it is an inexpensive technique it is prone to human error. This can include uneven distribution of cells, high cell densities, and different human perceptions of cell categorisation.[116] As manual cell counting can be a time-consuming task, especially for large cohort studies, Trypan blue can become toxic to cells over time and change the morphology of the cell, causing the influx of Trypan blue and identifying it as a dead cell.[117] This may have contributed to the consistently lower cell viability in the manual method compared to the semi-automated method. Although, a haemocytometer is highly reproducible, identification of live and dead cells is subject to variation in human

interpretation[118] and therefore reproducibility is subject to the user's degree of experience.

Semi-automated methods do not come without limitations. The frequency of misclassifying live and dead cells is higher compared the manual methods, such as with cell clumps, which can decrease the accuracy and percentage recovery.[118] This may explain the consistently lower total cell counts in the semi-automated method compared to manual methods. Semi-automated methods require pipetting which introduces the possibility of uneven distribution of cells.[116] Therefore, semi-automated methods may not reflect the true total cell count.

4.2.3 Cell counts and viability

The results from Trypan blue exclusion showed that there was a significant decrease in cells exposed to 1 mg/mL GTE, and no significant changes observed when exposed to 0.1 mg/mL GTE. There were no significant changes in total cell count (cells/mL). The results show that there was a dose-dependent change in cell viability and total cell count.

Similar occurrences of decreased cell viability or survivability have been demonstrated in other studies involving GTE or catechins. Kucera et al. observed a decline in cell viability and increase in MMP in primary rat hepatocytes over 24 h when exposed to 10, 30 and 100 μmol/L EGCG[65] doses 20 x lower than observed by Galati et al.[38] Lambert et al. conducted a closely related study showing a dose-dependent decrease in murine survivability upon exposure to EGCG, reaching up to 85% mortality.[51] However, a study involving sub-acute toxic doses of 625, 1250 and 2500 mg/kg GTE refuted these observations and found no

adverse pathological effects on mouse liver,[119] supported by another study at 1250, 2500 and 5000 mg/kg GTE.[120] Hepatotoxicity appears to be more severe in cases of pure catechin extract compared to GTE in both *in vivo* and *in vitro* techniques. However, this study is the first to find a significant change in cell viability using *in vitro* techniques.

The Trypan blue exclusion results are in direct contrast to the cell viability observed in the MTT assay. The results from the MTT assay showed that the viability increased in almost all instances compared to untreated cells with the exception of 50 μM EC. The MTT assay was attempted twice as the growth medium became too dark during incubation, which can affect spectrophotometer readings. The second attempt involved removing the spent medium and adding new medium prior to adding dye solution to reduce inaccurate absorbance readings, but may have caused loss of cells that did not adhere to the culture plate. The MTT assay has an advantage over the Trypan blue exclusion method in that it is more sensitive to detecting impairment of cell function, requires less labour and is quicker. However, recent reports have shown that free thiols and flavonoids in some herbal extracts may be involved in the reduction of MTT to formazan.[121,122] Other compounds that may interfere include GSH, ascorbic acid (vitamin C) and retinol (vitamin A_1).[123,124] Wang et al. also showed an underestimation in the antiproliferative activity of EGCG using an MTT assay over a 24h period.[125] Although MTT assays are considered the gold standard in cell viability testing, inconsistencies between the Trypan blue exclusion method and the MTT assay suggests that GTE, catechins and other phytochemicals may interfere with formazan reduction. Future studies in GTE hepatotoxicity may benefit from using a more appropriate assay to measure cell viability, such as the

adenosine triphosphate (ATP) or DNA assays, which have been shown to measure the true anti-proliferative action in green tea polyphenols.[125]

Trypsinisation is a commonly used procedure for detachment of cells from culture flasks and is arguably one of the best procedures for minimising cell damage and false negatives.[126] However, cells treated with 1 mg/mL GTE experienced difficulty in detachment from the culture plate when treated with PBS and 0.25% trypsin-EDTA. The experiment was repeated with adjustment to the amount of trypsin and incubation time, but with no improvement. This may be due to insufficient washing of cells with PBS, where it is known that calcium (Ca^{2+}) and magnesium (Mg^{2+}) in serum can cause cells to adhere together and to the culture flask.[116] However, studies have claimed that tea polyphenols, such as EGCG, have a non-competitive inhibitory effect on trypsin at a concentration of 0.05 mg/mL through conformational changes in the secondary structure of the protein.[127-129] Although not relevant in hepatotoxicity, this could be an additional biochemical pathway affected by GTE in the small intestine. Future studies may need adjustment to a higher concentration of trypsin and amount of PBS washes used.

4.2.4 Biochemical responses to green tea extract

Metabolomic analysis of GTE-treated HepG2 cells was conducted at two different concentrations (0.1 and 1 mg/mL) to establish possible biochemical pathways of GTE hepatotoxicity. Metabolites in cells exposed to 1 mg/mL GTE generally experienced a decrease in fold change the majority of the time, with amino acids being the most commonly identified class of compounds. Other compound classes detected include carboxylic acids, oxoacids, carbohydrates, nucleic and fatty acids. This contrasts with 0.1 mg/mL GTE exposure, where metabolites a more varied

change in response to exposure. This suggests that a change in GTE dosage may change the biochemical pathways affected, whether it be hepatotoxic or hepatoprotective effects.

4.2.4.1 1 mg/mL exposure

Amino acids

Amino acids are essential in the biosynthesis of proteins, DNA and carbohydrates. All identified amino acids in this study experienced a fold decrease, suggesting that amino acids were being catabolised. L-glutamine experienced the most substantial fold decrease upon exposure to 1 mg/mL GTE. L-glutamine is the active form of glutamine and a non-essential amino acid which has a crucial role in many processes in the liver, such as GSH biosynthesis.

GSH is an antioxidant used in many foods and supplements for protection against damaging oxidants. L-glutamine is converted to L-glutamic acid through the action of glutamine synthetase and combines with L-cysteine to form γ-glutamylcysteine. In the presence of glycine and GSH synthetase, γ-glutamylcysteine is then converted to GSH.[82] HepG2 cells experienced a fold decrease in both L-glutamine, L-glutamic acid and L-cysteine (Table 3.8), suggesting the rapid metabolism of these amino acids and an increase in GSH synthesis in the presence of GTE. This ultimately suggests that GTE exposure may be accelerating the production of GSH to counteract pro-oxidants, supporting the hypothesis of oxidative stress as a possible biochemical pathway of GTE hepatotoxicity. This is consistent with other *in vivo* liver studies, where there was an increase in ROS and increase of GSH in the presence of high doses of EGCG[38,65] or GTE.[130] However, some have suggested that changes in ROS

production and GSH occur depending on dose.[65,130] This further implies that GSH biosynthesis is a dose-dependent pathway for oxidative stress. This information potentiates the evidence of apoptosis occurring within the HepG2 cells; it is well-established that increased ROS production can trigger apoptosis through many avenues, such as p53 and caspase activities.[131] An intracellular ROS assay could be used to confirm high concentrations of ROS, in adjunct with TEM, to confirm apoptosis has occurred in GTE hepatotoxicity.

In surplus, the branched chain amino acids (BCAAs) identified in this study (L-isoleucine and L-valine) have been attributed to reduced oxidative stress[132] and hepatic apoptosis *in vivo*.[133] 8-oxoguanine DNA glycosylase 1 (OGG1) gene, encoding an enzyme involved in the removal of 8-oxoguanine residues formed from exposure to ROS,[134] was present in higher levels in liver injury-induced mice supplemented with BCAAs compared to healthy mice.[132] This suggests a possible avenue of ROS production at both a metabolomic and genomic level, where the metabolism of BCAAs may increase the availability of OGG1 and induce DNA repair in oxidative stress-induced cells, as suggested in Table 3.8 showing a fold decrease in uracil. Interestingly, Sugiyama et al. found that a higher concentration of BCAAs had an inhibitory effect on the growth of HepG2 cells, whereas lower concentrations did not suppress growth.[135] This contrasts with Figure 3.5, where the total cell count appeared to decrease upon exposure to 1 mg/mL GTE. This could indicate a dose-dependent response in cell growth, or the presence of other compounds contributing to stunted growth.

In conjunction with the BCAAs, the identified aromatic amino acids (AAAs) (L-tyrosine and phenylalanine) also experienced a fold decrease upon GTE exposure.

The Fischer ratio explains the relationship between decreased BCAA and increased AAA plasma levels during liver injury, specifically concerning hepatic encephalopathy.[136] This is thought to be due to increased BCAA catabolism within muscle cells and decreased AAA catabolism within the liver. As this study involved a hepatocellular carcinoma cell line and not an *in vivo* model, it limits the translatability to human models due to the absence of muscle cells, and therefore the study cannot follow the Fischer ratio. However, the Fischer ratio could aid in the diagnosis of systemic diseases such as hepatic encephalopathy upon exposure to GTE. As some GTE hepatotoxicity patients had experienced nervous system symptoms such as asterixis, it is valuable to further investigate the possibility of hepatic encephalopathy using *in vivo* models.

A more ambiguous explanation for GTE hepatotoxicity is the involvement of L-glutamine and L-glutamic acid in the urea cycle, also known as the ornithine cycle. L-glutamine is a precursor in the conversion of excess ammonia into urea within hepatic cells to allow excretion of highly-toxic ammonia.[82] It is not known whether deficiency in L-glutamine and L-glutamic acid directly induces excess ammonia levels in the liver. It could be suggested that glutamine synthetase, an enzyme that catalyses the production of L-glutamine from L-glutamic acid and ammonia, cannot exert its function due to low levels of its precursor molecules to cause the accumulation of ammonia in liver cells. Accumulation of ammonia and its metabolites (e.g. carbamoyl phosphate) has been shown to directly cause liver injury in those with urea cycle disorders,[137,138] but the exact mechanism has not been well characterised. It has also been suggested that ammonia exposure directly effects the mitochondrial RNA (mRNA) expression of antioxidant enzymes and pro-apoptotic genes, resulting in increased ROS production in *in vivo* settings.[139]

Oxoacids and other metabolites

Amino acids are not the only class compound that can contribute to GTE hepatotoxicity. The oxoacid phosphoric acid experienced a significant fold decrease upon exposure to 1 mg/mL GTE. Phosphoric acid is an important human metabolite appearing in many biological molecules, such as nucleic acids and glycerophospholipids. At a neutral pH, phosphoric acid can dissociate and exist as its conjugate bases dihydrogen phosphate and monohydrogen phosphate. A change in pH can form its conjugate base inorganic phosphate ion (P_i).[82] P_i is essential in the phosphorylation and dephosphorylation of cellular intermediates in metabolism, such as the phosphorylation of adenosine diphosphate (ADP) to ATP during oxidative phosphorylation. A deficiency in phosphoric acid may indicate high levels of P_i, suggesting that GTE may have changed the pH of the extracellular environment, causing a shift in the formation of P_i. This may cause uncoupling of oxidative phosphorylation from the ETC, influencing the MMP as seen by Kucera et al. and Galati et al.,[38,65] causing ATP depletion. It has been suggested that a collapsed MMP may be due to GTE-induced apoptosis,[38] but P_i deficiency may only be a minor contributor to this.

An interesting metabolite that was putatively identified was 1,2-benzenediol, also known as pyrocatechol. It is formed in the metabolism of L-tyrosine, and both experienced a fold decrease. The reduced presence of pyrocatechol in GTE-exposed cells is presumably due to stunted L-tyrosine metabolism, which reduces the production of intermediates involved in the TCA cycle,[82] contributing to ATP depletion. This is also evident in the significant fold increase of unidentified sugar_32.370, which may be a sugar involved in ATP synthesis. However, if

oxidative stress was contributing to ATP depletion, amino acid levels would be expected to be present in higher amounts compared to untreated cells. This is presumably due to insufficient ATP production, required for the biosynthesis of amino acids from TCA cycle and glycolytic intermediates.[140]

Analysis of the metabolic changes associated with 1 mg/mL GTE exposure suggests that the consumption of amino acids increases GSH and OGG1 production, as well as the possible accumulation of ammonia and its metabolites and ATP depletion. This, in turn, may cause increased ROS production and oxidative stress-induced apoptosis. Additionally, a systemic effect of GTE hepatotoxicity has been suggested if disease progressed. A summary can be found in Figure 4.1. Further studies into characterising the biochemical pathways of GTE-induced hepatotoxicity may include GTE exposure at multiple time points to estimate changes in metabolites in real time.

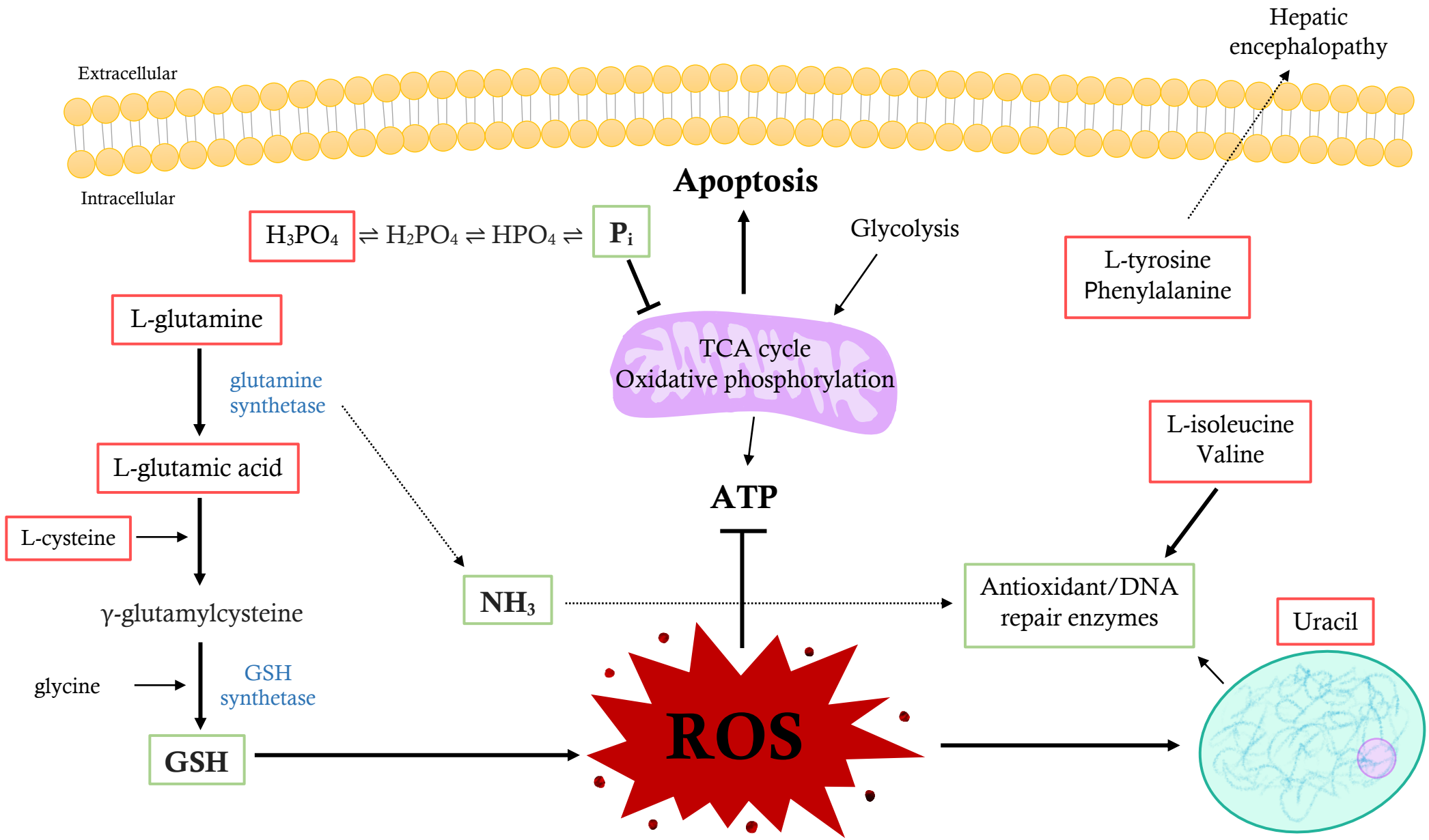

Figure 4.1. Schematic of proposed mechanism of GTE hepatotoxicity. Increased GSH is produced from the consumption of L-glutamine, L-glutamic acid and L-cysteine to neutralise ROS. ROS can damage DNA and cause ATP depletion and apoptosis. Decreased phosphoric acid (H_3PO_4) increases P_i, uncoupling oxidative phosphorylation. Decreased L-isoleucine, valine and increased ammonia (NH_3) may increase transcription of antioxidant and DNA repair enzymes. Decreased L-tyrosine and phenylalanine may cause systemic problems, such as hepatic encephalopathy. Key: red = decreased levels; green = increased levels.

4.2.4.2 0.1 mg/mL exposure

Amino acids

The composition of detected metabolites following exposure at 0.1 mg/mL was very different to what was observed upon 1 mg/mL GTE exposure. In the case of amino acids, the only identified amino acids were L-methionine, L-isoleucine and alanine, where three other amino acids were unable to be identified. L-isoleucine was the only amino acid in common with 1 mg/mL GTE exposure. According to statistical analyses, none of the fold changes were significant for amino acids.

Similar to 1 mg/mL GTE exposure, L-isoleucine had a 0.884-fold decrease in the presence of 0.1 mg/mL GTE. This suggests that L-isoleucine was being metabolised, however at a much lesser extent than at a higher GTE concentration. This could be attributed to stimulation of OGG1 gene production for ROS protection, however, the small fold change indicates L-isoleucine, along with L-methionine, was being metabolised for synthesis of TCA cycle intermediates for ATP production and protein synthesis.[140] The minor fold decrease in alanine was presumably due to pyruvate synthesis for gluconeogenesis. This is corroborated by the presence of three unidentified sugars, of which two experienced a mild fold increase.

Fatty acids and other metabolites

The only identifiable fatty acid was decanoic acid, also known as capric acid.[82] One unidentified fatty acid, unidentified fatty acid_32.275, had a 0.942-fold decrease that was significant from untreated cells. Fatty acids are heavily involved in β-oxidation, the catabolism of fatty acids to acetyl CoA, nicotinamide adenine

dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). These molecules are important intermediates or cofactors involved in the TCA cycle for ATP generation. The significant decrease in unidentified fatty acid_32.275 could be explained by the supplementation of fatty acids from the GTE, increasing β-oxidation of fatty acids. As previously stated, GTE supplementation may also induce PPAR genes involved in the up-regulation of β-oxidation in the liver.[101] This is conflicted by Kim et al., who suggested that GTE increases the mRNA expression of various lipogenic enzymes within the liver and that lipolytic and β-oxidation enzymes are not responsible for suppressing liver fat accumulation.[141] Another significant molecule was unknown_26.695, which experienced a 1.612-fold increase. However, barriers in metabolite identification prevented the molecule being identified. There were organic compounds that were also found that were unique to this data set, however are not involved in biological processes within HepG2 cells. The limitations of metabolite identification are discussed in section 4.3.

The limited number of significant fold changes upon exposure to 0.1 mg/mL GTE suggests that it does not have a significant effect on the biochemical pathways of hepatic cells at an acute dose. Its effectiveness into improving liver health is still questioned, but a chronic dose may be attributed to improving overall liver health. It has been suggested that chronic doses of GTE may aid in fat reduction[39] and anti-cancer effects.[40,41]

4.3 Limitations and future study

The study presented here is the first to look into the biochemical pathways involved in GTE hepatotoxicity at a metabolomic level. However, a major

limitation involving identification of the metabolites had the biggest impact on the outcomes. GC-MS has the advantage of having reproducible RTs compared to LC-MS, but the metabolome of different organisms and cell lines and the sheer number of metabolites within a database makes it difficult to identify the "correct" metabolite.[89] The NIST database, in particular, is a non-specific library that encompasses a whole range of chemicals including metabolites.[106] Therefore, compounds with a similar mass spectrum are more likely to be incorrectly identified. Scalbert et al. suggested that allowing a database to refine the search parameters for the spectral output, such as mammalian metabolites and toxins only, would improve the search results.[89] This would aid in filtering out chemicals involved in derivatisation, thus increasing the statistical power of multivariate analysis of these metabolites. Future studies may require cross-referencing mass spectra with multiple databases to increase the confidence of correct metabolite identification.

Beyond mass spectral databases, chromatographic techniques also contribute to limitations in metabolite identification. LC-MS, is valued for its sensitivity, ability to cover a wide range of compounds, giving the potential for comprehensive herbal medicine analysis, including challenging factors such as environment-dependent plant composition.[142] LC-MS provides information about the exact mass of a monoisotopic form of a compound, whereas GC-MS relies on the chemical derivatisation of compounds to increase volatility during analysis, which can hinder compound identification. *In silico* fragmentation methods have been suggested to improve the identification of unknown metabolites by predicting the fragmentation and RT of a compound using GC techniques, which has shown to detect the correct metabolite 73% of the time.[143] Ultimately, correct identification

of a metabolite is dependent on assessing chemical structure by comparison to reference data (i.e. the use of chemical standards), for which NMR spectroscopy is highly specialised for.[106] Therefore, future studies may include the use of NMR and targeted methods in order to confirm the presence and chemical structure of the metabolites identified in this study.

As with any *in vitro* study, the translatability to *in vivo* or human subjects is difficult due to the variability in an organism's metabolome. A single cell line can increase the reproducibility of metabolomic studies, but does not consider individual variability in metabolism and external factors, such as the coordination of other organs involved in metabolic pathways. The use of other hepatocellular carcinoma cell lines, such as HepaRG or primary hepatocytes, may aid in understanding the variability in the metabolome.

From this study, apoptosis has been suggested to contribute to GTE hepatoxicity. As previously mentioned, the use of TEM may provide visual indication of apoptosis to corroborate the evidence of the rounding of cells experienced upon exposure to 1 mg/mL GTE. This can also be corroborated with the use of apoptosis assay kits, such as the detection of loss of MMP experienced in the early stage of apoptosis as previously used in GTE hepatotoxicity studies.[38] Additionally, the use of ATP or DNA assays may be more appropriate to avoid the interference of formazan production by GTE during MTT assays.

5. Conclusion

Improving our understanding of GTE-related liver injury, which has been reported to be related to catechins, requires detailed biochemical investigation, which can be achieved using metabolomic analyses. This was applied in this project for the ability of metabolomics to characterise metabolites in a range of different GTE products and to determine their relative levels of different catechins in each product. Catechin levels varied widely among different GTE products, suggesting that changes in chemical composition of GTE products is associated with environmental conditions and manufacturing practices. Studies have suggested lethal doses of the catechins within *in vivo* models, and these variations may have implications in GTE hepatotoxicity. The other components of the GTE products were putatively identified and included fatty and amino acids. Many metabolites were identified to be chemicals used in the derivatisation process, and future studies may require utilisation of a greater number of mass spectral databases for correct identification.

Metabolomic techniques were also applied to characterise the biochemical pathways of GTE-induced hepatotoxicity in liver cells. GTE-exposed HepG2 cells responded in a dose-dependent manner, with decreasing structural integrity and cell viability. An MTT assay was used to confirm decreases in cell viability, but conflicted with Trypan blue results due to evidence of formazan production from flavonoids. Exposure to 1 mg/mL GTE induced more significant fold decreases in metabolites of HepG2 cells, including amino acids and oxoacids, compared to 0.1 mg/mL exposure. Changes in levels of these metabolites indicated that GTE

hepatoxicity is a dose-dependent process that may induce ROS production, ATP depletion and apoptosis, as corroborated by other studies.

Metabolomics is an emerging field that allows us to study the final downstream phenotype of an organism, identifying cellular pathways pertaining to abnormal changes in biochemistry. Although subsequent studies will require *in vivo* techniques and improvement in metabolite identification, this preliminary study further elucidated the biochemical pathways involved in GTE hepatotoxicity through novel metabolomic studies not yet employed in this research field. Additionally, this study contributed to determination of the chemical composition of different GTE products and characterisation of their changes in tea catechins levels; which may have implications in cases of hepatotoxicity.

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