Forensic application of nuclear versus mitochondrial DNA and the formation of cholesterol oxidation biomarkers in human cells after gamma-irradiation

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Abstract

Radiological crimes or terrorism could result in the exposure of biological evidence or individuals, including the perpetrators of the crime, to ionising radiation, such as gamma-radiation. Cellular damage via gamma-radiation is by direct ionisation, or more predominantly, by indirect oxidation of critical biomolecules due to reactive oxygen species (ROS) induction and oxidative stress. Subsequent degradation of deoxyribonucleic acid (DNA) evidence can impede genotyping efforts when the gamma-radiation dose is sufficiently high (> 1 kGy); this may impact mitochondrial DNA (mtDNA) more than nuclear DNA (nuDNA) due to its greater susceptibility to radiation-induced oxidation. Oxidation biomarkers are therefore candidate indicators of gamma-radiation exposure. This includes metabolic and ROS-derived cholesterol oxidation products (COPs), which are implicated in the oxidative cell stress response and as biomarkers for related pathologies. At gamma-radiation doses that are nonlethal or relevant to acute radiation syndromes (< 10 Gy), such biomarkers may have application for radiation injury triage, elimination of suspects in radiological crime, and elucidating mechanisms of cellular damage and adaptation.

This thesis consisted of two distinct objectives: (1) to compare the impact of high-dose (1 to 50 kGy) gamma-irradiation on the degradation of both nuDNA and mtDNA using forensic techniques; and (2) to evaluate the formation and biological significance of COPs after low-dose (1 mGy to 10 Gy) gamma-irradiation of human cells. For this purpose, a real-time polymerase chain reaction (PCR) protocol was developed for the evaluation of mtDNA degradation (Chapter 2), which was compared to that of nuDNA, after gamma-irradiation, based on autosomal short tandem repeat (STR) genotypes and indices of DNA integrity (Chapter 3). A method to quantify various sterols/COPs by gas chromatography-mass spectrometry (GC-MS) was also developed (Chapter 4) and applied to approximate their formation in gamma-irradiated cells (Chapter 5).

The real-time PCR method developed for mtDNA quantification comprised three human mtDNA targets of increasing length (86, 190 and 452 base pairs). This method was demonstrated to be capable of quantifying each target from $1 \times 10^4$ to 10 copies with a coefficient of determination ($R^2 \geq 0.999$) and includes an internal PCR control assay. The developed assays are human specific, sensitive (quantification limit of 10 copies), accurate (within 10 % relative error, for most target dilutions), and precise (within ± 10 % at 95 % confidence, for most target dilutions). Each target was used to calculate three mtDNA integrity indices comprising the
quantity ratio of intermediate/short, long/intermediate and long/short amplicons. This enabled comparison to peak height ratios for like-sized autosomal STR amplicons as indicators of DNA integrity after gamma-irradiation.

Gamma-irradiation improved GlobalFiler STR genotypes from liquid (hydrated) and dried (dehydrated) blood at lower doses, resulting in significant peak height increases at 1 to 5 kGy (by ~ 20-45 %) for hydrated samples and 1 to 10 kGy (by ~ 15-35 %) for dehydrated samples. This suggested potential for gamma-radiation to remove certain inhibitory effects. Degradation of STR genotypes was significant from 25 to 50 kGy (~ 30-70 % decrease in peak heights) for hydrated samples and at 50 kGy (~ 37 % decrease in peak heights) for dehydrated samples. However, full DNA profiles were attainable up to the highest dose examined of 50 kGy, with allelic dropout occurring for approximately 10 to 15 % of hydrated sample alleles at 50 kGy and most cases of subthreshold alleles being due to heterozygote peak imbalance.

From the calculation of integrity indices, gamma-irradiation of liquid and dried blood revealed that cell hydration exacerbated overall DNA damage as compared to desiccated cells, especially for mtDNA. Significant levels of degradation did not occur until 5 kGy for hydrated mtDNA and 10 kGy for hydrated nuDNA, while the same for dehydrated cells did not occur until 5 to 25 kGy for mtDNA (depending on the integrity index) and 25 kGy for nuDNA. Corresponding integrity indices were near 45 to 85 % lower for hydrated nuDNA and 55 to 90 % lower for hydrated mtDNA at 50 kGy. Significant differences between nuDNA and mtDNA integrity arose as early as after 1 kGy; dehydrated mtDNA had near 40 to 60 % lower integrity than dehydrated nuDNA, while hydrated mtDNA had near 30 to 75 % lower integrity than hydrated nuDNA, at 50 kGy. This was suggestive of increased oxidation originating from both water radiolysis and mitochondrial function. Given the persistence of more extensive damage to mtDNA than nuDNA when cells were dehydrated, albeit to an overall lesser extent than hydrated samples, residual moisture within air-dried cells may permit the same oxidative effects as hydrated cells, but in a restricted capacity. Other mechanisms may also exist to confer differential radiosensitivity between organelles, such as varied structural arrangement or frequency of ionisation events.

To monitor the formation of sterols/COPs after gamma-irradiation, methods were devised for the isolation and analysis of 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC and 27-HC, as well as the cholesterol precursor, lathosterol, by GC-MS. While the recovery of spiked sterols was about 10 % greater in the presence of cell matrix (~ 80-115 %
without matrix and ~ 90-125 % with matrix), cell medium reduced the linearity of calibration curves from a $R^2$ ranging 0.993-0.998 without matrix to 0.985-0.996 with matrix effects. Further, a loss in sensitivity was attained in the presence of cell medium, with quantification limits of 0.025 to 0.05 nanograms (ng) without matrix and 0.033 to 0.167 ng with matrix effects. However, the accuracy and precision of measurements was within 10-20 % for most analytes regardless of matrix type.

Production of potential biodosimetric markers (sterols/COPs) in peripheral blood mononuclear cells (PBMCs), as well as Jurkat cell lines, revealed a high degree of variation between replicates, indicative of variation due to the analysis method employed despite the suitable precisions previously ascertained. While PBMC sterols frequently resulted in change to measured concentrations greater than ± two-fold after irradiation, a near equal distribution of samples exhibiting increased and decreased sterol levels prevented any significance pertinent to biodosimetry. Jurkat cells did not demonstrate the same level of inconsistency in results, emphasising a potential susceptibility of PBMCs to matrix effects during analysis and/or interindividual variation in the sterol radiation response.

A significant and consistent hyperproduction of 24-HC and lathosterol in wild type Jurkat cells, near or beyond a 100 % increase of that for unirradiated control cells, was demonstrated at doses of 10 and/or 100 mGy. This effect was reversed when the mitochondrial translocator protein (TSPO) was overexpressed in Jurkat cells, resulting in significantly lower concentrations of 24-HC and lathosterol, near or beyond a 50 % loss relative to unirradiated control cells, at both 10 and 100 mGy doses. Thus, these sterols may function in the cellular adaptive response to radiation, representing potential biodosimetric markers for gamma-radiation exposure that is dependent on TSPO expression level. An increase in dose to 5000 mGy saw these effects diminish or reverse once more, suggesting higher doses or dose rates to impact COP biosynthesis / cell response, or a sustained response that is independent of the sterols examined. However, given large errors associated with measurements, this work requires repetition.

In summary, this thesis has demonstrated that elevated damage to mtDNA targets, such as the hypervariable regions (HVRs), can be expected after high-dose gamma-irradiation, as compared to nuDNA targets. However, at the doses examined, gamma-radiation alone is not sufficient to reduce the forensic efficacy of STR genotypes to the extent that HVR sequencing would be warranted. Thus, it is possible for STR genotyping to maintain practical value in a radiological
crime scene, at least where gamma-radiation is the only source of DNA damage. Furthermore, this thesis revealed that low doses of gamma-radiation do not elicit a correlative sterol response in human PBMCs that could be detected by the methods employed. Hence, the sterols extracted from these cells did not provide value for biodosimetry or radiation injury triage. However, the identification of consistent dose-dependent changes to sterol formation, isolated from cultured Jurkat cells, suggests potential for indicative changes in alternate human cell types that may differentially express TSPO. The sterols identified to respond to gamma-radiation are valuable targets for future studies of radiation biomarkers, as well as studies of cell death and adaptation to ionising radiation and/or oxidative stress.
Acknowledgments

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I would like to thank Shirani Katupitiya and Gregory Adcock of NCFS for their immense assistance with instrument troubleshooting and willingness to take time from running the laboratory to offer assistance whenever required. I’d also like to thank everyone at NCFS for their words of advice and feedback along the way. Thanks to James Robertson for acting as my administrative ‘primary’ toward the end!

For their assistance in the planning and implementation of gamma-irradiation work, as well as for reviewing the gamma-irradiation methodologies detailed in this thesis, I would like to thank Connie Banos and Justin Davies of ANSTO gamma-irradiation services. I would also like to thank Jennifer Harrison and Brett Rowling of the Institute for Environmental Research and Robert Russell of the National Deuteration Facility for permitting the use of instruments and laboratory space during developmental work. The Jurkat cell lines applied in this thesis were kindly provided by Richard Banati and Ryan Middleton of ANSTO Life Sciences. For the preparation and maintenance of Jurkat cell lines, I’d like to thank Guo Jun Liu.

I would also like to thank the Australian Federal Police Forensic and Data Centres for permitting me to carry out genotyping work using their equipment and laboratory, particularly Timothy Shaw for taking the extra time to perform the typing of profiles, as well as Paul Roffey for his friendly chats and advice beyond his role as a past honours supervisor.

The rollercoaster of this research ‘journey’ would have derailed if not for the strong support of my parents. Thank you for your love and encouragement – it’s my hope that you can now pronounce “mitochondria”. To Jade, Jake, and not least Jamin, thanks for all the welcome (and unwelcome!) distractions and your patience during the final stages.

Finally, this research would not have been possible without the support of my supervisory team: Dennis McNevin, Michelle Gahan, Andrew Wotherspoon and Guo Jun Liu. Thanks for your expertise, guidance, advice, mentoring, and for generally putting up with me over the years. It’s been a long ride, but thanks to you I have made it… with only slight scathes!
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<td>8-oxoG</td>
<td>8-oxo-7,8-dihydroguanine</td>
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<td>pK_a</td>
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<td>Acute radiation syndrome</td>
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<td>AP</td>
<td>Apurinic/apyrimidinic</td>
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<td>APCI</td>
<td>Atmospheric pressure chemical ionisation</td>
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<td>ANTARES</td>
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<td>bp</td>
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<tr>
<td>Bq</td>
<td>Becquerel</td>
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<tr>
<td>SN₂</td>
<td>Bimolecular (second order) nucleophilic substitution</td>
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<td>CT</td>
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<td>COP</td>
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<td>Double strand break</td>
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<td>Double-stranded DNA</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>EI</td>
<td>Electron ionisation</td>
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<td>Electron volt</td>
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<td>Electropherogram</td>
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<tr>
<td>fg</td>
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<td>IED</td>
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<td>IND</td>
<td>Improvised nuclear device</td>
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<td>IPC</td>
<td>Internal PCR control</td>
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<td>IAEA</td>
<td>International Atomic Energy Agency</td>
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<td>$^{192}$Ir</td>
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<td>LET</td>
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<td>LC</td>
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<td>Liquid-liquid extraction</td>
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<td>LXR</td>
<td>Liver X receptor</td>
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<td>Mg$^{2+}$</td>
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<td>MSD</td>
<td>Mass selective detector</td>
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<td>MIDAS</td>
<td>Mitochondrial DNA absence sensitive factor</td>
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<td>MAX</td>
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<td>MSTFA</td>
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<td>N-trimethylsilylimidazole</td>
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<td>NGM</td>
<td>Next Generation Multiplex</td>
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<td>⁶⁰Ni</td>
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<td>PAT</td>
<td>Peak amplitude threshold</td>
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<td>PFB</td>
<td>Pentafluorobenzoyl</td>
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<td>POP</td>
<td>Performance-Optimised Polymer</td>
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<td>Peripheral blood mononuclear cell</td>
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<td>PCR</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
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<td>KOH</td>
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<td>C&lt;sub&gt;q&lt;/sub&gt;</td>
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<td>RED</td>
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<td>Radiofrequency</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>rRNA</td>
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<td>Roswell Park Memorial Institute</td>
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<tr>
<td>s</td>
<td>Second</td>
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<td>SIM</td>
<td>Selected ion monitoring</td>
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<td>STR</td>
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<td>Single-stranded DNA</td>
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Publications


Presentations

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Ethics statement

All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approval for the collection and use of human biological material was granted by the University of Canberra Committee for Ethics in Human Research (Project Number 14-70). All biological material was collected with the informed consent of the donors. This thesis does not contain any animal studies.
Chapter 1: Introduction
1.1 Current radiological threat

The atrocity of 11 September 2001 have seen to global change in the national security apparatus. Increased funding and legislative changes have enhanced counterterrorism intelligence to diminish the potential for future attacks (Grono 2004). The war on terrorism led by the United States has resulted in losses to the leadership, manpower and finances of al-Qaeda and its networks (Enders & Sandler 2005; O’Brien 2011), destabilising the established hierarchical structure of transnational terrorism (Sofer 2011). Although the emergence of the self-proclaimed Islamic State has seen some restoration to this structure of terrorism, a large-scale, logistically complex attack is now considered a task of immense difficulty to emulate (Enders & Sandler 2005; Sofer 2011).

Contemporary terrorism is ‘online’, relying heavily on social networks to acquire sympathisers within Western societies (O’Brien 2011; Sofer 2011; Weimann 2004). The result is a shift in terrorism trends to favour more frequent, but less complex attacks (Enders & Sandler 2005; O’Brien 2011). The less predictable disposition of small-scale attacks poses a more complicated challenge to intelligence efforts, and therefore such ‘lone wolf’ attacks are now the greatest threat. Further, it has been known for some time that terrorist organisations have an expressed interest in acquiring and implementing unconventional weaponry, such as those of a chemical, biological or radiological nature for use as weapons of mass destruction (Daly et al. 2005; McCloud et al. 2005; Rising 2006). Sydney terror plotters linked to Islamic State were caught in 2017 with plans for the construction of a hydrogen sulphide bomb (Zhou 2017). However, it was the 2014 theft of non-fissile uranium from Mosul University in northern Iraq that implied an interest of Islamic State militants in the use of radiological weapons (Nichols 2014). Subsequent to this, Twitter boasts by jihadist sympathisers painted London as a potential target for a dirty bomb (Dorman & Doyle 2014).

Dirty bombs are a type of radiological dispersal device (RDD) that combine conventional high explosives with radioactive material. A dirty bomb is designed to spread radioactive contamination in an overt manner; ‘dilution’ of radioactive material outside of the dispersion epicentre limits immediate casualties akin to a conventional improvised explosive device (IED). Contrary to popular misconception, dirty bombs therefore lack the destructive power (fission-based nuclear explosion) of an improvised nuclear device (IND). Further, they are a less efficient cause of acute radiation toxicity than is the covert dispersal of a radioactive aerosol or the placement of a sealed radioactive source, or radiation emission device (RED) (Bushberg et
However, this public perception and fear of radiation bolsters the effectiveness of the explosive RDD for radiological terrorism, making the dirty bomb, a weapon of mass disruption, the most likely device to be implemented in a radiological attack (Colella et al. 2005; Karam 2005; Leikin et al. 2003; Medalia 2003; Steinhausler 2003). Furthermore, the complexity and technical expertise required to design an IND, as well as acquire and maintain fissile material, makes an RDD, unlike an IND, a very real threat (Wirz & Egger 2005).

While a RDD attack has not yet taken place, they are not a newly founded concept. Chechen rebels were the first to plant explosive RDDs, though none were detonated, both in Moscow 1995 (Specter 1995) and Chechnya 1998 (Isayev 1998). In 2002, José Padilla (a.k.a. Abdullah al Muhaqir), following meetings with al-Qaeda, was apprehended for conspiring to construct and detonate a RDD within the United States (Risen & Shenon 2002). In 2003, British intelligence had purported al-Qaeda to have manufactured a dirty bomb within Afghanistan and produced a report suggesting an attack was imminent, which prompted the United States Government to search for RDDs in several cities later in 2003 and early 2004, though none were uncovered (Krikorian 2004) (Johnson 2003). Another set of terror plots were disrupted after the 2004 arrest of Dhiren Barot (a.k.a. Issa al Britani) and several other al-Qaeda affiliates, which included the planned bombings of targets in the United Kingdom and United States using RDDs (Gardham 2006; Isikoff & Hosenball 2004). Although, it is unclear if al-Qaeda had ever held such a device, the interest of such groups in radiological terrorism remains a certainty.

1.1.1 Ionising radiation

Radiation is the energy released, as subatomic particles or electromagnetic waves, by atoms with excess energy. Electromagnetic radiation is produced from oscillating electric and magnetic fields; such is generated upon absorption of energy by an atomic electron, where the emission of electromagnetic waves, carried by photons, returns the electron to its original, lowest energy state (Liou 2002). Contingent on atomic properties and the amount of energy received, the frequency of oscillations will differ to release distinctive forms of electromagnetic radiation. Most forms, such as radiowaves, microwaves, infrared, visible and ultraviolet (UV) light, are nonionising, that is, they do not contain enough energy to completely liberate electrons from atomic orbit to result in an electrostatically charged atomic state, i.e. ionisation (Ng 2003). However, higher frequency (shorter wavelength) electromagnetic waves do have
sufficient energy to ionise matter and therefore disrupt molecular bonds, including gamma- and X-radiation. Particle radiation is also capable of causing ionisation, which is released from highly unstable atomic nuclei, including alpha, beta, proton and neutron radiation; these may also be associated with an electromagnetic emission.

Ordinarily, protons and neutrons (collectively nucleons) are held together within an atomic nucleus by the strong nuclear force, which overpowers the electromagnetic force that would otherwise repulse the like-charged protons, as reviewed by Lilley (2001) and Martin (2013). As the number of protons increases, changing the type of atom, so too does the repulsive electromagnetic force; this must be offset by a growing number of neutrons in order to supply additional binding energy to maintain the strength of the nuclear attraction. The laws of nature also impel the arrangement of these nucleons toward the lowermost attainable potential energy. When this is not achieved, due to a disproportion in the nucleon composition, the nuclide will contain excess energy and be unstable (radioactive). Such radionuclides undergo radioactive decay, emitting ionising radiation in the form of energetic subatomic particles and/or electromagnetic radiation (Table 1.1), transforming into a more stable configuration.

Table 1.1: Particle properties of common forms of ionising radiation.

<table>
<thead>
<tr>
<th>Radiation type</th>
<th>Particle description</th>
<th>Atomic mass units</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha decay</td>
<td>Helium nucleus (2 protons + 2 neutrons)</td>
<td>4</td>
<td>+ 2</td>
</tr>
<tr>
<td>Beta minus decay</td>
<td>Electron</td>
<td>1/1823</td>
<td>– 1</td>
</tr>
<tr>
<td>Beta plus decay</td>
<td>Positron (antimatter electron)</td>
<td>1/1823</td>
<td>+ 1</td>
</tr>
<tr>
<td>Proton emission</td>
<td>Proton</td>
<td>1</td>
<td>+ 1</td>
</tr>
<tr>
<td>Neutron emission</td>
<td>Neutron</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>X-ray</td>
<td>Photons (wavelength 10 nm – 10 pm)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gamma-ray</td>
<td>Photons (wavelength 10 pm – 1 pm)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

nm = nanometres; pm = picometres

The type of particle released depends primarily on the size of the atom and the direction of the nucleon imbalance, as reviewed by Lilley (2001), Martin (2013) and Podgorsak (2006). Proton-rich radionuclides (atomic number > 82), in general, decay by releasing an alpha particle, identical to a helium nucleus. Alternatively, a proton of a lighter radionuclide can be converted into a neutron to emit a positron, the antiparticle of an electron, a form of beta decay. Neutron-rich radionuclides can transform a neutron into a proton to result in the ejection of an electron, another type of beta particle. Proton and neutron emissions only occur in nature from cosmic
sources where the corresponding imbalance is extreme, otherwise attainable only by artificial means. Gamma-ray photons may accompany alpha and beta decay in order to expel any remaining excitation energy necessary to return the nucleus to its lowest energy state. X-ray photons are produced either as electrons transition to fill vacancies incurred in lower orbitals, or as bremsstrahlung (braking radiation), produced when beta particles are decelerated by electromagnetic interaction with atomic nuclei.

The mass and charge of each particle dictates its ionising and penetrative power by influencing its linear energy transfer (LET). LET, as defined by Park and Kang (2011), describes the amount of energy deposited by, or lost from, a particle per unit distance or track length, measured in kiloelectron volts (keV; Table 1.2) per micrometre (μm). As ionising particles expend energy through their interactions with matter, they lose kinetic energy and are eventually halted. An LET below 10 keV/μm is considered ‘low’ and such radiation is sparsely ionising, maintaining its velocity for longer, while an LET above this threshold is considered ‘high’ and is densely ionising and rapidly impeded. The LET of each type of ionising radiation will vary inversely to its initial kinetic energy, a product of its mass, as well as the mechanism of particle interaction with matter.

Table 1.2: Definitions of specialised radiation-specific units and terminology.

<table>
<thead>
<tr>
<th>Unit</th>
<th>Definition and related terminology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Becquerel (Bq)</td>
<td>A measure of activity (radioactivity), the number of radionuclides that decay (undergo nuclear transformations) per time. The activity per mass of radionuclide (Becquerel per gram; Bq.g⁻¹) is known as the specific activity. The time required to halve the number of radionuclides, and thus activity, is the radioactive half-life (t½).</td>
</tr>
<tr>
<td>Electron volt (eV)</td>
<td>A measure of the energy acquired by an electron that moves through an electrical potential difference of one volt.</td>
</tr>
<tr>
<td>Gray (Gy)</td>
<td>A measure of absorbed dose, the energy absorbed per mass of matter.</td>
</tr>
<tr>
<td>Sievert (Sv)</td>
<td>A measure of equivalent dose, the absorbed dose weighted for the biological impact of the radiation involved. Equivalent (1:1) to the gamma-radiation absorbed dose. The equivalent dose weighted for tissue sensitivity is the effective dose.</td>
</tr>
</tbody>
</table>

References: Bushberg et al. (2007); IAEA (2007); Turner (2007)
Charged particles (alpha, beta, proton) are considered to be directly ionising in that they interact with orbital electrons via direct electromagnetic forces and readily interact with matter to rapidly impart energy (Podgorsak 2006). The large size of alpha particles cause them to travel with the lowest initial velocity, and combined with their highly reactive double charge, they are considered high LET particles (Loucas et al. 2012; Wild et al. 2011), which cannot penetrate the epidermis. Due to their negligible mass, beta particles have a lower LET and are able to move up to several metres (m) through air and centimetres (cm) into tissue. Although a beta source could cause burns to the skin if left in contact, alpha- and beta-emitters are most hazardous if presented internally via ingestion, inhalation or passed through an open wound. The penetrability of radiation particles and associated risks as health hazards are reviewed by Bushberg et al. (2007), Veenema and Karam (2003) and Wild et al. (2011).

Neutral particles (neutrons, photons) ionise matter indirectly by generating secondary charged particles within the medium, which govern ionisation through direct electromagnetic interactions. The release of a charged particle can be achieved through several processes (e.g. photoelectric and Compton effects, elastic scattering, neutron capture), resulting in the emission of electrons/positrons or protons in the case of photon or neutron interactions, respectively (Nelson & Reilly 1991; Park & Kang 2011). While neutrons carry mass, photons travel at the speed of light, having no mass or charge; they therefore do not readily interact with matter and thus embody low LET radiation (Loucas et al. 2012). In actuality, LET has no meaning for photons since their absorption can take place in a single interaction, but the secondary charged particles produced can be described by LET (Nelson & Reilly 1991). Subsequently, gamma-radiation has low ionising power, but is able to penetrate many metres through air as well as tissue, and is a hazard if a source is presented externally as well as internally to the body (Veenema & Karam 2003).

Other forms of ionising radiation also exist, emitted during the processes of electron capture, internal conversion and spontaneous fission. Fission reactions compete with alpha decay and involve the emission of heavy particles as large atoms (atomic number > 92) split into two fission fragments, releasing enormous amounts of energy. This same process is exploited for nuclear reactors and weaponry; however, unlike nuclear (induced) fission, spontaneous fission is not a self-sustaining chain reaction (Podgorsak 2006).
1.1.2 Radioactive materials for a dirty bomb

Radioactive material is far more widely available than nuclear fissile material, allowing material for a RDD or RED to be more easily acquired than that for an IND. This is because of the increasing and widespread use of commercial radioactive sources in medicine and industry (e.g. radiotherapy, industrial radiography) that are under less stringent security than are sources in nuclear power or research facilities, especially in nations lacking strict regulation of such materials (IAEA 2005; Yoo et al. 2011). These sites offer an opportunity for illicit procurement through theft or hijacking of shipments, which may be carried out by individuals with access to such material for reasons concerning extortion, bribery or ideology (Ferguson et al. 2005).

In the period from 1993 to 2017, 913 incidents of undetermined intent were reported to the International Atomic Energy Agency (IAEA), most of which involved the theft or loss of nuclear or radiological material (IAEA 2018). The detection and recovery of uncontrolled sources, such as from unauthorised disposal or desertion of such material, has contributed to a further 2044 incidents. Another 278 incidents of confirmed trafficking or malicious use were reported. The former IAEA chief portrayed these activities as an ongoing and aggravated issue that may lead to radiological terrorism (Golovnina 2012). These incidents highlight the availability of sources for appropriation by individuals with malicious intent; however, for a radioactive substance to be useful for a RDD, and thus pose a high security threat, it must meet explicit criteria.

First, a potential source must be prevalent in medicine and/or industry so that it can be readily acquired. It must then be portable, that is, able to be contained with relative simplicity for transport and manoeuvrability in order to mitigate exposure to the perpetrator, although this is less important for a suicide attacker. The source must be in a form that is easily dispersed, such as a powder/dust/aerosol, which can be ingested and inhaled to maximise the toxicity from alpha- and beta-emitters. Finally, it must have a long half-life to capitalise upon the disruptive economic impact from contamination, although not so long as to reduce the activity to negligible levels; thus, it must exhibit high levels of radioactivity in low/diluted quantities to increase its potential for radiotoxic effects (i.e. a high specific activity).

These criteria are reviewed by Ferguson et al. (2003) and Ferguson et al. (2005), as are seven commercially available reactor-produced radioisotopes that meet these requirements. These are the alpha-emitters, americium-241 ($^{241}$Am), californium-252 ($^{252}$Cf) and plutonium-238 ($^{238}$Pu), and the beta-emitters, caesium-137 ($^{137}$Cs), cobalt-60 ($^{60}$Co), iridium-192 ($^{192}$Ir) and strontium-
90 (\textsuperscript{90}Sr). Of these radioisotopes, high-energy gamma-emissions are also associated with the decay of \textsuperscript{137}Cs, \textsuperscript{60}Co and \textsuperscript{192}Ir, with their ability to serve as external hazards augmenting their internal biological damage. The most common gamma source for irradiation studies is \textsuperscript{60}Co.

\subsection{Cobalt-60}

Radioactive \textsuperscript{60}Co is a product of neutron capture (Park & Kang 2011), where a linear accelerator is used to bombard monoisotopic cobalt-59 (\textsuperscript{59}Co) with neutron radiation; the absorption of a neutron by the stable \textsuperscript{59}Co nucleus yields the radionuclide (Moss & Yaffe 1953). It decays with an approximate specific activity of 4.19\times10^{13} \text{Bq/g} (41.9 \text{terabecquerels (TBq)/g}) and half-life of 5.27 years to stable nickel-60 (\textsuperscript{60}Ni), via a principal low energy beta-emission (electrons), followed by almost equally abundant high energy gamma-emissions (Figure 1.1) (Karam 2005; Moss & Yaffe 1953; Tuli 2003). Where beta-radiation is present, bremsstrahlung X-rays will also be produced (Boehm & Wu 1954). The typical activity of \textsuperscript{60}Co sources in medicine and industry range from $8.7\times10^{-4}$ to $1.5\times10^{5}$ TBq, while a minimum activity of 30 TBq would likely be life threatening proximal to the dispersion epicentre, owing to the short exposure time required to receive a lethal dose (IAEA 2005; Shin & Kim 2009). However, with increased distance and shielding, and reduced exposure time, the biological radiation effects would be mitigated.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cobalt-60.png}
\caption{Cobalt-60 (\textsuperscript{60}Co) to nickel-60 (\textsuperscript{60}Ni) radioactive decay scheme. Only beta minus ($\beta^{-}$) and gamma ($\gamma$) emissions above 0.1 \% abundance are shown. With an initial heightened energy level of 2824 kiloelectron volts (keV), \textsuperscript{60}Co decays with a half-life of 5.27 years, primarily via a $\beta^{-}$ emission with an energy of 318 keV to an excited state of \textsuperscript{60}Ni. The remaining 2506 keV is reduced to a stable level by subsequent $\gamma$ emissions of 1173 and 1332 keV. Adapted from Tuli (2003).}
\end{figure}

The applications for \textsuperscript{60}Co include sources for sterilisation and food preservation, self-shielded
and blood/tissue irradiators, radiotherapy, industrial radiography, calibration, as well as various level and converyer gauges (IAEA 2005). In addition to its long half-life and potential for high activity, $^{60}\text{Co}$ is highly prevalent, and the small size of these sources and containment within appropriate shielding allows them to also satisfy the requirement for portability (Medalia 2003). Sources of $^{60}\text{Co}$ are typically manufactured solid metal discs, pellets or pins, which could be dispersed as shrapnel within a RDD, although its synthesis as a cobalt oxide powder provides a much greater capacity for dispersion (EPA 2006; Shin & Kim 2009). However, the highly penetrating $^{60}\text{Co}$ gamma-rays, with a typical LET of 0.3 keV/μm (Park & Kang 2011), ensure its threat as a contaminant external to the body, even if its metal form renders internal contamination impractical.

### 1.2 Forensic DNA analysis

Investigation of radiological crimes, inclusive of not only a radiological terror incident, but also the unregulated disposal, theft or trafficking of radioactive substances, may rely on the availability of deoxyribonucleic acid (DNA) evidence for conclusive victim and/or perpetrator identification. This is achieved through genotyping, a process that allows for the determination of known genetic variants (alleles) at set DNA target regions (loci) that, when taken together, are particular to an individual. In a forensic DNA investigation, a reference genotype belonging to a known individual is matched against an unknown genotype. The probability of a random match between any two matched genotypes is estimated using statistical genetics based on known allele frequency data, and the strength of the match defined by a likelihood ratio (Collins & Morton 1994; Kaplow 2014).

The forensic DNA analytical process typically begins with extraction of DNA from biological samples (e.g. hair, blood, skin cells) and removal of contaminants, such as proteins and lipids, via a DNA purification step. The DNA targets are then quantified and amplified via polymerase chain reaction (PCR) to increase their copy number to a detectable level. Each distinct amplified target must then be separated for their individual detection to generate the genotype. One of many complications for genotype interpretation is DNA degradation, a common phenomenon for crime scene samples, but particularly so in disaster victim identification (DVI) settings, such as would follow a terrorism incident. Forensic targets can include both nuclear (nuDNA) and
mitochondrial DNA (mtDNA), with differing discrimination power and ability to facilitate identifications from degraded DNA.

1.2.1 DNA structure

The nucleic acid, DNA, is composed of two antiparallel chains of base-paired deoxyribonucleotides (Figure 1.2) (Crick & Watson 1954; Watson & Crick 1953a). The phosphate backbone is negatively charged and is responsible for the polyanionic property of DNA, which engages it in strong electrostatic interactions; counter-ions (e.g. sodium and potassium cations) in the aqueous cellular environment are required to neutralise the negative charge in order to stabilise the typical double helix structure of DNA in cells (Denisov & Halle 2000). The molecule is then compacted into chromatin and the chromosome structure through supercoiling and association with histone proteins in the cell’s nucleus (Margueron & Reinberg 2010).

![Polynucleotide Chain](image1)

![Nitrogenous Base](image2)

**Figure 1.2: Polynucleotide and base structure of DNA.** Each nucleotide is attached to contiguous nucleotides by a phosphodiester bond at the 3'- and 5'-carbon atoms of the deoxyribose sugar; new nucleotides are added to the 3'-hydroxyl terminus so that DNA synthesis occurs in the 5'→3' direction. A 1'-N-glycosidic bond connects the sugar to the base nitrogen for base-pairing between two antiparallel strands. Hydrogen bonding from adenine (A) to thymine (T) and guanine (G) to cytosine (C) holds the complimentary strands together. Adapted from Watson and Crick (1953b).
1.2.2 Forensic DNA targets

1.2.2.1 Nuclear DNA and short tandem repeats

A single nucleus is present within each cell and houses the single maternal and paternal copies of linear chromosomal DNA (autosomal and sex chromosomal), totalling around 3.2 billion base pairs (bp) in humans. Targets suitable for forensic genotyping include only polymorphic loci, which differ with high frequency between individuals. Current practice involves typing of length polymorphisms in repetitive DNA, termed microsatellites or short tandem repeats (STRs). STRs are noncoding loci that contain a repeating core nucleotide sequence (1-6 bp) that varies between alleles in the number of repeats (Kimpton et al. 1993). Repeating sequences of four (tetra-) or five (penta-) nucleotides are routinely applied for forensic genotyping. Dozens of alleles can exist for a single STR, lending substantial statistical and probative value to identifications when multiple STRs are targeted simultaneously (multiplexed).

The Combined DNA Index System (CODIS) defines targets required for upload to DNA criminal databases in the United States. This originally included 13 core autosomal STR loci, which has been expanded as of 1st January 2017 to include 20 autosomal STRs. These are targeted by the AmpF/STR GlobalFiler PCR Amplification Kit (Applied Biosystems). The Australian National Criminal Investigation DNA Database (NCIDD) currently includes 17 core STRs plus amelogenin (Wilson-Wilde et al. 2013), comprising all 13 original CODIS loci and four of the extended CODIS loci. These markers are included in both the PowerPlex 21 (Promega) and GlobalFiler chemistries. The United Kingdom’s National DNA Database (NDNAD) currently includes 12 European Standard Set (ESS) loci, which overlap with current CODIS loci. These are targeted, together with three additional CODIS loci, by the AmpF/STR Next Generation Multiplex (NGM) PCR Amplification Kits (Applied Biosystems) (ENFSI DNA Working Group 2017). Such STRs, when taken together, can provide random match probabilities in the range of one per trillion or less (Budowle et al. 2000; Hares 2015). Thus, autosomal STRs are the current standard for forensic identity testing, together with part of the X-Y homologous amelogenin gene for sex-indication (Sullivan et al. 1993).
1.2.2.2 Mitochondrial DNA and hypervariable regions

The cell’s mitochondria provide an alternate source of DNA for forensic application. The maternally inherited circular mitochondrial genome is 16,569 bp in length according to the revised Cambridge Reference Sequence (rCRS); this is the current human reference for determination of mtDNA sequence polymorphisms, such as nucleotide substitutions, insertions or deletions (Anderson et al. 1981; Andrews et al. 1999). Studies of the rCRS have revealed a noncoding ‘control region’ (displacement loop, D-loop) encompassing nucleotide positions 16024-576, a site of frequent sequence polymorphism (Lott et al. 2013). Polymorphisms within the control region are localised primarily around two hypervariable regions (HVRs); HV1, which is most polymorphic at nucleotide positions 16024-16365 (subregion HV-I); and HV2, which is most polymorphic at positions 73-340 (subregion HV-II) and 438-574 (subregion HV-III). Of the three subregions, HV-III is least polymorphic (Lutz et al. 1998; Mabuchi et al. 2007). Mabuchi et al. (2007) reported HV-I and HV-II to give a combined random match probability of around 1.00 %, with inclusion of HV-III imparting only a 0.01 % improved difference, which is vastly inferior to that of multiplexed STRs (Section 1.2.2.1). Thus, HV-III is generally excluded from HVR analyses given the added analysis costs with little gain.

The primary benefit of HVR sequencing can be attributed to its high copy number per cell. Multiple (2-10) copies of the mitochondrial genome reside within each of the hundreds to thousands of mitochondria per cell; thus, depending on cell type and proliferation stage, there is potential for several thousand copies per cell (D’Erchia et al. 2015; Trinei et al. 2006). Consequently, mtDNA offers a greater opportunity for successful genotyping of HVRs from a degraded DNA source or limited sample. For samples suffering from high DNA degradation, HVR sequencing is often able to be applied for identity testing when STRs are unreliable or unavailable. Exclusive maternal inheritance of mtDNA enables reference material to be obtained from maternal relatives when a direct reference source is inaccessible and offers potential to reveal maternal lineage of a DNA donor.

1.2.3 DNA extraction

Before DNA evidence can undergo forensic analysis, it must first be liberated from the biological substrate via a cell lysis and DNA purification/concentration technique. A chemical lysis procedure is most often employed, utilising reducing agents (e.g. dithiothreitol (DTT)), detergents (e.g. sodium dodecyl sulphate (SDS), Triton X), chaotropic salts (e.g. guanidinium
thiocyanate/chloride) and/or digestive enzymes (e.g. protease, proteinase K) to dissociate lipid and protein membrane interactions and denature DNA digestive enzymes (nucleases). Metal chelators (e.g. ethylenediaminetetraacetic acid (EDTA), chelex resin) may also be included to remove divalent metal cations (nuclease enzymatic cofactors) from solution. Cell lysis can also be accomplished by mechanical, thermal or electrical means, which can be applied alone or in conjunction with chemical lysis procedures (Kim et al. 2009; Tan & Yiap 2009). Extracted/purified DNA can be dissolved in a buffer solution of Tris-chloride (pH 8) with EDTA (TE buffer).

Purification of DNA from lysed cellular material is necessary to remove compounds that may interfere with downstream applications (e.g. PCR), which may include chemicals added during the lysis procedure, and to concentrate the DNA into a suitable volume for such applications (Demeke & Jenkins 2010). Numerous techniques exist for this purpose, such as filtration (e.g. centrifugal filters), solid-phase extraction (e.g. silica resins, paramagnetic beads), and solvent-based and salting-out/precipitation approaches (e.g. phenol-chloroform extraction, alcohol precipitation) (Kim et al. 2009; Tan & Yiap 2009). The prevailing methods are commercial solid-phase approaches due to ease of automation, high throughput and avoidance of hazardous solvents. Silanol groups (SiOH) of silica resin-based spin columns are dehydrated in the presence of chaotropic salts and/or alcohol to bind dehydrated DNA via a salt cation bridge while most contaminants are non-retained. The silica membrane is washed with low salt buffer to remove residual contaminants and maintain DNA binding. Alcohol is applied to wash out salts before DNA elution under low ionic strength and high pH TE buffer, ensuring deprotonation of silanol groups and electrostatic repulsion of DNA from the silica (Tan & Yiap 2009).

Traditional phenol-chloroform (organic) DNA extractions are considered highly efficient for pure DNA recoveries from difficult, low-template samples, such as decomposed remains, hair or bone (Allen-Hall & McNevin 2012; Köchl et al. 2005; McNevin 2016; McNevin et al. 2005; Mirmomeni et al. 2010). This approach acts to remove contaminating lipids and proteins owing to solubility differences in a biphasic solvent system; DNA partitions into an aqueous phase, lipid dissolves into an organic phase, and proteins are retained at the biphasic interface (Tan & Yiap 2009). An alcohol precipitation, commonly in 65-75 % (v/v) ethanol, can be applied to remove contaminating polar solutes from the aqueous phase in the presence of salt, commonly 0.3 molar (M) sodium acetate (Chen et al. 2010). Absolute ethanol lowers the polarity of aqueous solution to dehydrate both DNA phosphate anions and salt cations to enable
electrostatic attraction, removing the DNA and salts from solution as polar insoluble precipitates (Piskur & Rupprecht 1995). The precipitate is isolated by centrifugation and the pellet washed in 70 % ethanol to remove salts before being redissolved and concentrated in TE buffer (Tan & Yiap 2009).

1.2.4 Polymerase chain reaction (PCR)

PCR is DNA replication that takes place in vitro, where a DNA target (amplicon) is copied by a cyclical reaction that aims to double the number of target copies at the end of each cycle, producing billions of copies after 30-40 cycles. This is achieved in the presence of a thermostable DNA polymerase (commonly Taq), together with magnesium cofactor (Mg\(^{2+}\)), primers, and deoxyribonucleotide triphosphates (dNTPs), by strict temperature control. A PCR comprises three steps governed by temperature: (1) denaturation, where the two DNA strands are dissociated into single strands (typically near 95 degrees Celsius, °C); (2) annealing, where single-stranded primers hybridise to sequences flanking the target region (typically near 60 °C); and (3) elongation/extension, where DNA polymerase incorporates dNTPs onto the primers to lengthen them across the target region (template), creating a complimentary copy of the template (typically near 75 °C). This technique forms a pivotal part of forensic DNA genotyping, enabling approximation of DNA quantity in an unknown sample (DNA quantification), as well as the isolation and preparation of forensic DNA targets for detection (DNA amplification).

1.2.4.1 DNA quantification

Genotyping of forensic targets is optimised for an explicit DNA quantity within a specified volume. Determination of DNA quantity, and quality, is therefore essential for the selection and preparation of suitable samples prior to genotyping. Quantification of DNA extracts can be carried out by techniques including UV spectrophotometry, fluorometry, chemiluminescence, agarose gel electrophoresis and PCR (Demeke & Jenkins 2010; Nicklas & Buel 2003).

For downstream applications involving PCR, DNA should be quantified with a PCR-based approach that is able to depict the concentration of amplifiable (intact) DNA, as well as verify the presence of inhibitors. Real-time quantitative PCR (qPCR) can differentiate between low-template and inhibited samples by the inclusion of an internal PCR control (IPC), a synthetic
oligonucleotide that is amplified alongside the DNA extract, where the absence of IPC amplification indicates the presence of PCR inhibitors. Another advantage of qPCR is the ability to target human-specific loci, which can include male-specific and sex-unspecific loci, permitting the omission of DNA from food-stuff, animal or bacterial contamination, as well as differentiation between male and female DNA (Nicklas & Buel 2003). For this reason, qPCR is the prevailing technique applied for DNA quantification within forensics.

For quantification by qPCR, amplified product is detected at the end of each PCR cycle in real-time by the incorporation of a fluorogenic probe or intercalating dye. The resultant fluorescence intensity is directly proportional to the number of target molecules present at the end of each cycle (PCR product). Quantification via qPCR probes is commonly achieved via a two-step PCR, whereby the annealing and extension steps are carried out at the annealing temperature to prevent the probe from melting off the template. The amplification consists of three phases: (1) the exponential phase, where PCR product doubles with each cycle; (2) the linear phase, where the reaction slows as reactants (Mg\(^{2+}\), dNTPs, primers) begin to deplete, and (3) the plateau phase, where at least one of the reactants has been completely exhausted and thus fluorescence remains constant with each subsequent cycle. A threshold cycle (C\(_T\)) or quantification cycle (C\(_q\)) is set during the exponential phase, which describes the cycle number required for signal to exceed background fluorescence or noise. Quantification is achieved from the C\(_q\) based on a linear plot of standards of known concentration (standard curve).

A common type of fluorogenic probe is the TaqMan probe, which is utilised by Applied Biosystems’ Quantifiler chemistries. Probe fluorescence, when intact, is suppressed by a proximal quencher dye that absorbs any visible light emitted by the fluorophore. The TaqMan assay (Figure 1.3) involves target-specific binding of probes to template, between the two flanking PCR primers, with no fluorescence until the emitter and quencher are separated as a result of extension at the end of each PCR cycle (Nicklas & Buel 2003). This differs from intercalating dye chemistries, such as SYBR Green I (Figure 1.3), a minor groove binder, in that fluorescence is quenched while the dye is in the free state and induced when the dye is bound to DNA, with a higher affinity to bind double-stranded (ds)DNA than single-stranded (ss)DNA (Dragan et al. 2012; Nicklas & Buel 2003). The non-specificity of intercalating dyes means that they do not target any particular sequence and may therefore exaggerate fluorescence intensity if other sources of DNA are present, most notably, primer-dimers.
Chapter 1

1.2.4.2 DNA amplification

High copy numbers of target loci are a requirement for genotyping in order to meet the sensitivity of detection methods; this is achieved by PCR (Section 1.2.4). Additionally, PCR is a convenient means to hybridise onto amplicons a fluorogenic probe/dye or other molecule/sequence required by downstream analysis methods. This is achieved by incorporating these molecules onto the 5′-ends of PCR primers. Multiple targets can be amplified with the use of separate PCRs for each amplicon (singleplexed) or by a single PCR that amplifies all targets simultaneously (multiplexed), which minimises sample handling, reagent consumption and analysis time, but is more complicated to achieve.

Multiplexing is a prerequisite for genotyping of the dozens of forensic STRs. This requires PCR primers for each target to have a similar annealing temperature, with detection of different amplicons by size (Section 1.2.5.1). For convenience and standardisation, pre-optimised commercial STR kits have been developed. Assays applied by the forensic community for current database requirements include the 24-plex GlobalFiler PCR assay, the 21-plex PowerPlex 21 System, and the 17-plex NGM SElect PCR assay (Section 1.2.2.1). These kits feature amplicons ranging around 80 to 450 bp in length, depending on the allele present and primer sites of different kits. These kits each include several ‘mini-STRs’ (< 200 bp) that assist genotyping of degraded template (Section 1.2.6.2).
1.2.5  Amplicon detection

1.2.5.1  Capillary electrophoresis

Electrophoresis is a technique used to separate charged molecules by their mass as they move through a semi-porous matrix. The polyanionic nature of DNA causes it to be repelled from a negative electrode (cathode) and migrate toward a positive electrode (anode). As the molecules move through the matrix, commonly a gel, the movement of high molecular weight fragments is hindered more substantially than is low molecular weight fragments. High resolution separation of amplicons permits detection of alleles that differ in size by a single base pair, or base in the case of denatured (single-stranded) amplicons (Buel et al. 1998; Moretti et al. 2001).

Capillary electrophoresis (CE) involves the migration of denatured amplicons through a thin fused-silica capillary column (Figure 1.4), replacing the gel with a semiliquid polymer, but otherwise operating on the same principles as gel electrophoresis (Buel et al. 1998). The commercial polymer, Performance Optimised Polymer (POP)-4 (Applied Biosystems), is commonly used for STR analysis, containing 4 % polydimethylacrylamide, 8 M urea and 5 % 2-pyrrolidinone (Rosenblum et al. 1997). The cathode and anode are submerged within separate buffers at the capillary inlet and outlet, respectively. Amplified DNA fragments are concentrated onto the capillary column via an electrokinetic injection, where a voltage is applied to the anode to create an electric current between the sample and the positive electric field of the anode buffer for a specified time. The anionic DNA fragments, suspended in a highly deionised (Hi-Di) formamide denaturant to prevent injection competition and ensure single strands, are then electrostatically attracted from within the sample into the capillary column (Butler 2012).

Since amplicons are distinguished by size, those within an overlapping size range can be detected via the hybridisation of a 5′-non-nucleotide linker to modify amplicon size and/or a 5′-fluorophore label of varying colour. These are attached to the primers applied during the PCR (Section 1.2.4.2). As labelled amplicons pass through a detection window, fluorescence is induced by an excitation source that is captured by a multiwavelength detection system (charge-coupled device, CCD) to produce a multicoloured electropherogram (EPG) (Butler et al. 2004).
Figure 1.4: **Capillary electrophoresis.** Amplicons labelled with a 5′-fluorophore travel toward the anode buffer through a thin-fused silica column packed with a semiliquid polymer that retards the migration of larger amplicons. As size-separated amplicons pass through a detection window, their fluorescence is recorded as a distinct signal on an electropherogram. Adapted from Butler (2012).

The EPG depicts the alleles identified at each locus as peaks measured in relative fluorescence units (RFU), separated by size and fluorophore colour. Allele sizes are calibrated by co-electrophoresis of an internal size standard of known fragment sizes, and unknown alleles are labelled by comparison to an allelic ladder of known alleles (Butler et al. 2004). Peak amplitude thresholds (PATs), including analytical and stochastic thresholds, dictate the minimum RFU for detection and match reporting of alleles, respectively. The analytical or detection threshold is the minimum RFU at which a peak can be considered above the background signal/noise of the instrument, being a possible true allele. The stochastic or match threshold is the minimum RFU at which allele dropout is unlikely to cause false reporting for a single-source profile. A stochastic threshold is set for homozygous loci, where the same allele is inherited from both parents (i.e. single peak at a single locus), and heterozygous loci, where two different alleles are inherited (i.e. two peaks at a single locus). These thresholds are empirically determined by the laboratory according to guidelines such as SWGDAM (2017).
1.2.5.2 **Hypervariable region sequencing**

Genotyping of the mitochondrial HVRs differs from that of the autosomal STRs in that, instead of fragment length polymorphisms, genotyping of the HVRs concerns sequence polymorphisms by comparison to the rCRS. For this reason, the detection approach must be modified so that each individual base at each position of the target sequence can be identified. This is commonly achieved by utilising fluorophore-labelled irreversible- or reversible-terminator dNTPs during the PCR, which are colour-coded for each base (Chidgeavadze & Beabealashvilli 1984; Turcatti et al. 2008). Non-optical approaches are also emerging, such as sequencing by pH (voltage) changes as each base is sequentially added to the reaction (Rothberg et al. 2011). Sanger (chain-termination) sequencing (Sanger et al. 1977) using gel- or capillary-based electrophoresis is the most effective of the first-generation techniques and retains superior accuracy to massively-parallel sequencing (MPS; next-generation sequencing) array-based approaches (Heather & Chain 2016). However, MPS offers lower cost and faster speed for high-throughput applications, being able to sequence millions of molecules simultaneously without electrophoresis (Grada & Weinbrecht 2013; Liu et al. 2012).

1.2.6 **Interpretation**

Analysis of genetic profiles firstly involves the assignment of alleles in comparison to reference genotypes and evaluation of stochastic EPG artefacts that may compromise the validity of any result. For STR genotyping by PCR and CE approaches, potential artefacts can include high stutter peaks, heterozygote peak ratio imbalance, pull-up, null-alleles and/or allelic dropout/drop-in (Butler et al. 2004; Gill et al. 2015; Pompanon et al. 2005). An increase in stochastic effects, especially allelic/locus dropout, is of most concern to low-template/degraded DNA. The complexity of interpretations is increased for mixture analysis (multiple contributors to the DNA substrate), which can be deconvoluted by various computational approaches such as STRmix (Bright et al. 2018; Bright et al. 2016), TrueAllele (Perlin et al. 2013; Perlin et al. 2011), or R packages (Haned & Gill 2011; Tvedebrink 2011), as examples. Once alleles have been assigned, statistical evaluation is performed for any two matched genotypes.
1.2.6.1 Statistical genetics: Likelihood ratio

The probability that any two random members of a population share an allele at a particular locus is vital to determining the true value of a forensic genotype. It is therefore necessary for allele probability data to be gathered for each of the alleles of an STR kit for diverse populations, such as the Australian population study for PowerPlex 21 loci by Bright et al. (2014). This enables the strength of a genotypic match to be defined. To weight or quantify the extent of a match based on known allele probability data, likelihood ratios are employed, which describe the ‘times more likely’ that a genotype has come from the examined individual rather than from a random member of the population (Gill et al. 2015; Morrison 2011; Stockmarr 1999). With allelic random match probabilities of ≤ 10 %, this value can exceed $1 \times 10^{20}$ when 20 loci are matched. For low-template/degraded DNA evidence, likelihood ratio models exist that account for the presence of stochastic genotyping effects, such as dropout (Steele et al. 2014).

1.2.6.2 Effects of DNA degradation on genotyping

The DNA molecule can be subjected to physical and/or chemical attack resulting in the breakdown of the DNA molecule. Genetic damage can be introduced by enzymatic digestion from endogenous or exogenous nucleases, spontaneous hydrolysis or oxidation, or exposure to adverse environmental conditions such as intense heat, UV or ionising radiations (Alaeddini et al. 2010; Lindahl 1993). Forensic DNA evidence is frequently degraded by such sources, which presents problems for genotyping by PCR.

Successful PCR requires that the template remains intact; the presence of damaging lesions within the template and/or primer binding sites may impede DNA replication through prevention of strand uncoiling, misrecognition of primers, and/or blockage of DNA polymerase. Failure of the PCR at any of these steps will prevent amplification of some or all alleles for genotyping (Alaeddini et al. 2010). Base misincorporation by DNA polymerase can also result from certain miscoding lesions; e.g. resultant G·C→A·T transition after hydrolytic deamination of cytosine to uracil (Hofreiter et al. 2001). By way of containing a greater number of sites for nuclease, chemical, or physical interaction, longer amplicons are more frequent targets for damage than shorter amplicons (Takahashi et al. 1997). As a result, genotypes of degraded DNA are characterised by dropout of alleles belonging to loci of larger size ranges, generating a ‘decay curve’ in the EPG. The greater the extent of degradation, the greater this effect, which
will gradually impact shorter alleles (Chung et al. 2004).

Several strategies exist that attempt to mitigate the effects of DNA degradation. Recent studies have evaluated standard and/or modified procedures for restoration of AmpF/STR Identifiler or Identifiler Plus genotypes using the PreCR Repair Mix, a cocktail of base excision repair enzymes, with varied success (Ambers et al. 2014; Diegoli et al. 2012; Robertson et al. 2014; Sim et al. 2011). Use of alternative or multiple polymerases with the ability to proofread (high-fidelity) or extend template over blocking lesions has also been considered (D’Abbadie et al. 2007; Hansen et al. 2001). A more routine and validated procedure for analysis of degraded DNA is the implementation of reduced size amplicons (for STRs and HVRs), achieved by selecting primer binding sites closer to the informative target region (Lee et al. 2008a; Mulero et al. 2008; Wiegand & Kleiber 2001). Shorter amplicon size ranges allow for a greater extent of degradation to be tolerated before its effects on genotyping are observed (Chung et al. 2004).

Given the differential influence of amplicon size on the amplification efficiency of degraded DNA targets, qPCR approaches are a useful means to assess the extent of degradation of samples prior to genotyping. This is achieved by implementing multiple amplicons of increasing size; an index of DNA degradation is found as a ratio of short versus long amplicon quantities, which is expected to rise as the quantity of longer targets becomes diminished due to ‘preferential’ degradation (Holt et al. 2016; Swango et al. 2006). Even without incorporation of multiple amplicons, DNA degradation can be monitored relative to a non-degraded control, if available.

The ability to profile STRs from forensically relevant biological matrices, after various high doses of alpha- (0 to 26,400 kilogray, kGy) and gamma-irradiation (0 to 100 kGy), was examined in the study of Abbondante (2009). A typical EPG degradation pattern was displayed after alpha-irradiation at and beyond 66 kGy and gamma-irradiation exceeding 1 kGy, with potential for full-profiles up to 10 kGy gamma-irradiation. As demonstrated, the less densely ionising but more penetrative gamma-ray is a more efficient DNA degradative source than the alpha-particle, at least when considering the capacity for STR genotyping of forensic samples. In contrast to Abbondante (2009), the impact of gamma-radiation on both STR genotypes from bloodstains and HVR sequencing from hairs has been demonstrated to have little effect up to 90 kGy (Monson et al. 2018). However, the requirement for substantial doses (> 1 kGy) is apparent in order to degrade forensic targets, where it is understood that ionising radiation elicits greater damage to mtDNA than nuDNA (Sections 1.3.1 and 1.3.2.1). The effects upon
forensic nuDNA and mtDNA targets have not been comparatively demonstrated after collection from the same cell matrix.

1.3 Biological effects of ionising radiation

Chemical and functional modifications to critical biomolecules such as DNA, proteins and lipids precede cellular abnormalities or cell death from ionising radiation (Reisz et al. 2014). This biomolecular damage is sustained via direct energy deposition to the critical biomolecules themselves or to surrounding cellular constituents (predominantly water). Indirect cell damage due to radiation then proceeds via secondary chemical mediators, namely free radicals, that form as a result of primary ionisation events (e.g. water radiolysis, lipid peroxidation), changes to cellular oxidative capacity, or subsequent free radical reactions. While critical damage from direct action is most frequent from high LET radiations, these interactions are only accountable for 30-40 % of biological damage from low LET radiations (Michaels & Hunt 1978). The remaining 60-70 % of cell damage is incurred by indirect action, i.e. the resultant oxidative stress.

1.3.1 Reactive oxygen species and oxidative stress

Oxidation is an electron transfer reaction involving electron loss; the counter reaction, reduction, involves electron gain. Oxidants (electron acceptors) have a high potential to be reduced, thereby driving oxidation of susceptible targets. The group of oxidants derived from molecular oxygen (O₂), reactive oxygen species (ROS), encompass both free radicals (e.g. superoxide ('O₂⁻), hydroxyl radical ('OH)), which contain one or more unpaired valence electrons, and non-radicals (e.g. hydrogen peroxide (H₂O₂)) that are themselves oxidants and/or can be converted into radicals (Halliwell 2006). The hydroxyl radical is most biologically reactive, being extremely short-lived (half-life of 10⁻⁸ to 10⁻⁹ seconds) and reacting almost immediately with proximal molecules to generate the majority of oxidative damage (Buettner 1993; Dorfman & Adams 1973; Halliwell & Whiteman 2004). Superoxide and hydrogen peroxide derive their cytotoxicity from Haber-Weiss and Fenton chemistry to propagate hydroxyl radical formation (Benov 2001; Mello Filho et al. 1984). Free radical reactions are therefore the main contributor to oxidative cell damage (Figure 1.5).
Cells generate ROS under normal physiological conditions primarily as byproducts of mitochondrial oxidative metabolism via premature electron leakage from the electron transport chain (ETC) (Halliwell 2006; Selivanov et al. 2011; Turrens 2003). The continuous generation of ROS byproducts within the mitochondria will appreciably elicit greater oxidative damage to this organelle (Kowaltowski & Vercesi 1999). This has been highlighted by studies that have revealed oxidative damage to be more extensive in mtDNA than nDNA in untreated cells, which was increased by induction of oxidative stress and by gamma-irradiation (Morales et al. 1998; Richter et al. 1988; Yakes & van Houten 1997). The implication of this on mtDNA profiling success in cases involving degraded nDNA is uncertain (Section 1.2.6.2). This effect has been attributed to the signalling of damage between adjacent mitochondria, resulting in the upregulation of ETC function and mitochondrial content via cell cycle arrest and accumulation of cells in the G2/M phase, which has been observed for both beta- and X-irradiated cells up to 10 Gy (Leach et al. 2001; Yamamori et al. 2012). In addition to increased mitochondrial ROS, ionising radiation is also able to generate ROS and other reactive species via water radiolysis (Figure 1.6).
Figure 1.6: Generation and reactions of reactive oxygen species (ROS) from ionising radiation leading to biomolecular damage. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), act to defuse the ROS, superoxide (\(\cdot \text{O}_2^-\)) and hydrogen peroxide (\(\text{H}_2\text{O}_2\)), in low quantities by their conversion to water (\(\text{H}_2\text{O}\)). ROS propagation by ionising radiation-induced upregulation of electron transport chain (ETC) function and water radiolysis can overwhelm antioxidants to induce a state of oxidative cellular stress. Biomolecular damage is governed by oxidative reaction primarily via the hydroxyl radical (\(\cdot \text{OH}\)); the additional water dissociation products, atomic hydrogen (\(\cdot \text{H}\)) and solvated/hydrated electrons (\(\text{e}_{\text{aq}}^-\)) elicit biomolecular damage through their reactions as reductants (Azzam et al. 2012; Das 2013; Halliwell 2006; Turrens 2003).

Intracellular water comprises 70-80 % of the mammalian cell mass (Delgado et al. 2013) and is thus a prime target for direct interaction with ionising radiation (Azzam et al. 2012; Michaels & Hunt 1978; Yamaguchi et al. 2005). While hydroxyl radicals and the reductants, hydrated/solvated electrons (\(\text{e}_{\text{aq}}^-\)) and atomic hydrogen (\(\cdot \text{H}\)), are the major water dissociation products produced in response to low LET radiations, radiolytic yields shift to favour non-radicals, such as hydrogen peroxide and molecular hydrogen (\(\text{H}_2\)), as LET increases (Le Caër 2011). Hydrated electrons are able to form anionic biomolecular subunits and further reactive products from water (Alizadeh et al. 2013). In the presence of oxygen, hydrated electrons and atomic hydrogen are rapidly converted to superoxide and its conjugate acid, the perhydroxyl (\(\cdot \text{HO}_2\)) radical, which dissociates to superoxide at physiological pH (Azzam et al. 2012; Buettner 1993; Michaels & Hunt 1978).
Low-level ROS production is involved in cell signalling and regulation in processes such as cell growth, differentiation and autophagy, enzyme activation, inflammation and other immune functions, as well as controlled, apoptotic cell death (Finkel 2011; Scherz-Shouval & Elazar 2007; Wu & Bratton 2013; Yang et al. 2013). However, the non-specific reactivity of ROS causes them to be biologically damaging if their production is left uncontrolled. Mechanisms of ROS protection have evolved to mitigate the oxidative damaging effects in low quantities, including antioxidant defence (Birben et al. 2012), damage removal and replacement systems (Davies 2000), and DNA repair (Lu et al. 2001). When these processes are deficient, or are overwhelmed by ROS, a state of oxidative cell stress can arise.

Oxidative stress follows an excess generation of prooxidant species in relation to antioxidant species, leading to unconstrained oxidative cellular damage (Sies 1997). Upregulation of defensive systems may be the first response of cells to rising ROS (Halliwell 2006). This ‘overprotection’ or ‘adaptive response’, described as the enhanced resistance of cells to the damaging effects of a toxic agent after a low dose pre-exposure (Crawford & Davies 1994), may then precondition the cell for subsequent, increased levels of ROS. At sublethal stress levels, a form of cytostatic senescence, characterised by irreversible replicative cessation, can accompany these changes (Burova et al. 2013; Chen & Ames 1994). As oxidative damage increases, cell constituents and defences may become irreversibly impaired, requiring autophagic intracellular digestion and replacement (Filomeni et al. 2015), or initiation of cell death (Circu & Aw 2010; Gardner et al. 1997; Hampton & Orrenius 1997; Saito et al. 2006). Cell death may take place with characteristics of both apoptosis and necrosis (Higgins et al. 2012; Tan et al. 1998).

1.3.2 Biomolecular damage

The capacity for a cell to carry out its physiological role is dependent upon its functional integrity at a macromolecular level (Reisz et al. 2014). DNA damage has long been recognised as the primary mechanism by which the cytotoxic effects of ionising radiation are incurred (Iyer & Lehnert 2000; Munro 1970a; Munro 1970b; Radford 1986). This is due to a reliance on DNA integrity for turnover of lipids and proteins via protein biosynthesis (inclusive of protein machinery for lipid biosynthesis), as well as for DNA self-replication. Unlike lipids and proteins, damaged DNA must therefore be repaired and cannot be simply replaced (Davies 2000; Lu et al. 2001; Sancar et al. 2004). Thus, while a small amount of DNA damage (to a
single molecule) can be catastrophic to the cell, a much greater level of damage to lipids or proteins can be tolerated. For the cell to sustain injury from lipid or protein damage, the entire lipid or protein network must become compromised, or at least, pass a threshold level of damage, which is indeed the case following self-propagating oxidative cellular modifications (Benderitter et al. 2003; Haimovitz-Friedman et al. 1994; Kiang et al. 2012; Krisko et al. 2012; Mishra 2004; Schneiderman et al. 2001). The damages caused by ionising irradiation of DNA (Section 1.3.2.1) and lipid networks (Section 1.3.2.2) are described.

### 1.3.2.1 DNA damage

Ionising radiation damages DNA via both its direct and indirect (oxidative) actions. Direct excitation or ionisation of DNA constituents (phosphate, base, sugar) localises electron gain (reduced/anionic) and electron loss (oxidised/cationic) centres within the DNA molecule, causing structural modifications by electron or proton transfer reactions (Cullis & Symons 1986). Interaction of short-lived hydroxyl radicals with proximal DNA constituents accounts for indirect damage via addition reactions with base double bonds and hydrogen abstraction from base and sugar residues (Balasubramanian et al. 1998; Cannan & Pederson 2016; Dizdaroglu et al. 2002). Together, these modes of damage lead to the accrual of various lesions within the DNA molecule (Figure 1.7).

**Figure 1.7: DNA lesions induced by ionising radiation.** (1) Abasic site and hydrogen bond breakage; (2) base oxidation; (3) double strand break with overlapping ends (4) 3’phosphate (blocking moiety); (5) intrastrand crosslink; (6) interstrand crosslink; (7) crosslinking protein; (8) sugar residue alteration; (9) single strand break; (10) base deamination.
DNA lesions may be induced by a variety of reactions. Formation of new covalent bonds gives rise to interstrand, intrastrand, and DNA-protein crosslinks (Dextraze et al. 2010; Stingele & Jentsch 2015). Sugar hydrogen abstraction and sugar radical-mediated oxidation may produce strand breaks and base liberation (Close et al. 2013; Pogozelski & Tullius 1998). Free base release from glycosidic bond breakage produces abasic, or apyrimidinic/apurinic (AP) sites, which are highly reactive alkali-labile residues that lead to strand breakage under alkaline conditions (Lhomme et al. 1999). Single strand breaks (SSBs) involve the partial fragmentation of the DNA molecule caused by breakage of phosphodiester bonds, with or without nucleotide release, along one of the two DNA strands. Complete fragmentation across both DNA strands may arise from two proximal opposing SSBs or other clustered lesion, replication fork stalling (e.g. from blocking lesions, abasic sites or crosslinks), or attempted repair of clustered damage (Cannan & Pederson 2016; Cullis & Symons 1986; Dextraze et al. 2010). These double strand breaks (DSBs) often fragment unevenly to produce overlapping ends with 3′-blocking moieties, such as 3′-phosphate or 3′-phosphoglycolate, impeding replication (Lees-Miller & Meek 2003).

The low redox potentials of DNA base residues, especially of guanine, leads to base oxidative damage from ROS interaction. While cytosine and thymine are able to undergo oxidation to hydantoins (Madugundu et al. 2014), the most commonly encountered marker of DNA oxidation is 8-oxo-7,8-dihydroguanine (8-oxoG) (Krzysztof et al. 2013; Steenken & Jovanovic 1997). When present, the 8-oxoG lesion is able to base pair with adenine to cause G·C→T·A transversions during replication or PCR, and can be further oxidised to imidazolone that is able to base-pair with guanine to cause G·C→C·G transversions (Kino & Sugiyama 2001). Approximately eight times more 8-oxoG lesions were induced in rat liver mitochondria (0.53 picomole (pmol) per microgram (µg) mtDNA) than nuclei (0.068 pmol/µg nuDNA) after 150 Gy gamma-irradiation (Richert et al. 1988), accentuating the role of mitochondrial ROS in DNA oxidative damage from ionising radiation (Leach et al. 2001; Yamamori et al. 2012).

Since high LET radiation is densely ionising, direct damage and highly clustered lesions are frequent. Clustered/complex lesions are multiply damaged sites, where two or more DNA lesions are incurred within a single helical turn (~ 10 bp) by a single radiation track (Nikjoo et al. 1999). Low LET radiation, being sparsely ionising, mediates most damage via indirect routes, primarily as a result of hydroxyl radical reactions, producing less frequent clustered damage (Yokoya et al. 2009). About 60 % of strand break and base damage is present in clustered form for low LET radiation with up to 10 lesions per cluster, while this value rises to 90 % for high LET radiation with up to 25 lesions per cluster (Cannan & Pederson 2016;
Nikjoo et al. 1999). Further, for low LET radiation, it is estimated that about 750 SSBs and 50 DSBs are sustained per Gy per cell, while for high LET radiation, these values are about 680 and 160, respectively (Semenenko & Stewart 2004). These damages hinder in vivo and in vitro DNA replication, which become less efficient with increasing LET (Matuo et al. 2013).

Placement of lesions is considered to be mostly random for ionising radiations; however, a non-random distribution of high LET damage lesions has been linked to the packaging of DNA into chromatin structures (Cannan & Pederson 2016; Semenenko & Stewart 2004). Chromatin may condense into heterochromatin in order to inactivate (silence) genes, but must decondense into euchromatin to give way to mediators of transcription, replication, or repair. The occurrence of DSBs, as implied by the formation of DSB repair foci, phosphorylated histone H2AX (γ-H2AX), has been shown to preferentially form in euchromatin (Cannan & Pederson 2016; Vasireddy et al. 2010). This is a possible consequence of damage exacerbation by enzyme access to clustered lesions during attempted replication/repair.

### 1.3.2.2 Lipid peroxidation

Lipids are a diverse class of biomolecule that are insoluble, or partly insoluble, in water (‘fat’ soluble), consisting of a hydrocarbon backbone. Many lipids contain long carboxylic acid (COOH) tails termed fatty acids that are esterified onto a more polar lipid head group, including the fat storage lipid triacylglycerol, cellular membrane phospholipids, and cholesterol (Abbott et al. 2012). Fatty acids differ in the length of their aliphatic chains and their saturation in single carbon-carbon bonds; most unsaturated bonds refer to double carbon bonding, resulting in bends in the aliphatic structure. When integrated onto membrane lipids, these bends prevent tight packing to increase membrane fluidity; specifically, fluidity is increased when four or more unsaturated bonds are present (Yang et al. 2011). For this reason, polyunsaturated fatty acids (PUFAs) are often incorporated onto membrane lipids and, by virtue of their double bonds, are most susceptible to peroxidation (Kris-Etherton 1999; McIntyre & Hazen 2010; Yun & Surh 2012).

Lipid peroxidation is self-propagating (Figure 1.8), initiated by abstraction of weakly bonded allylic hydrogens and perpetuated until termination by antioxidants or free radical self-conjugation (Ayala et al. 2014; Kiang et al. 2012; Montuschi et al. 2004; Reisz et al. 2014; Repetto et al. 2012). An allylic hydrogen is defined as being bound to an allylic carbon, which is itself defined as being bound adjacent (via single bond) to a doubly bonded carbon. The
bisallylic hydrogens contained within PUFAs are the most vulnerable groups to peroxidation, giving rise to sensitive markers of oxidative stress status, such as the prostaglandin-like F₂-isoprostanes, derived from arachidonic acid (Ayala et al. 2014; Montuschi et al. 2004; Roberts & Morrow 2000; Yin et al. 2003). While hydroxyl radicals react rapidly with such vulnerable groups, propagation of longer-lived peroxyl or alkoxyl radicals within lipid bilayers provide greater opportunity for peroxidation of less susceptible membrane lipids (that lack bisallylic hydrogens), such as cholesterol (Murphy & Johnson 2008).

![Lipid peroxidation of unsaturated fatty acids.](image)

**Figure 1.8: Lipid peroxidation of unsaturated fatty acids.** Lipid peroxidation is initiated by radical-mediated abstraction of a bisallylic hydrogen from a lipid molecule (RH), leaving a carbon-centred alkyl radical (R•) (1). The alkyl radical is stabilised by rearrangement of the double bond to form a conjugated diene (2). A lipid peroxyl radical (ROO•) is then formed by addition of molecular oxygen (O₂) (3). The peroxyl radical stabilises as a lipid hydroperoxide (ROOH) by propagating hydrogen abstraction and alkyl radical formation (4). The hydroperoxide may decompose in the presence of transition metal catalysts (Fe²⁺) to alkoxyl radicals (RO•) (5), which may abstract hydrogen to stabilise as lipid alcohols (6). Alcohols or alternative stable arrangements (aldehydes, ketones, alkanes) may be cytotoxic or can further the radical chain reaction. Adapted from Ayala et al. (2014) and Repetto et al. (2012).
1.3.2.2.1 Cholesterol oxidation products

Cholesterol is a steroid alcohol (sterol) characterised by the presence of a carbon-3 (C3) hydroxyl (alcohol) group on its steroid ring structure, with a C17 aliphatic (isooctyl) side chain. Cholesterol oxidation can take place within its hydrocarbon ring or aliphatic tail to form a variety of cholesterol oxidation products (COPs; oxysterols). These compounds may be derived from ROS/radical reaction (autoxidation) or enzymatically/metabolically (Figure 1.9). The ring structure contains a C5 double bond, such that its peroxidation is initiated predominately by abstraction of the C7 allylic hydrogen for formation of peroxyl or alkoxyl radicals (Murphy & Johnson 2008). Thus, C7 ring-oxidised COPs are the major cholesterol autoxidation products, namely 7β-hydroxycholesterol (HC) and 7-ketocholesterol (KC) (Gill et al. 2008). Metabolic oxidation of cholesterol occurs through the action of cholesterol hydroxylases, most of which are members of the microsomal and mitochondrial cytochrome P450 superfamily (Nebert & Russell 2002). These typically act upon the aliphatic cholesterol side chain (24-, 25-, 27-HC), with the exception of some ring-oxidised COPs (4β-HC and 7α-HC). Additionally, some COPs can be produced through both pathways, such as 7α-HC and 25-HC (Gill et al. 2008).
Figure 1.9: Structures of cholesterol (5α-cholesten-3β-ol) and some of the major oxysterols and their origins. Most oxysterols are derived from cytochrome P450 family (CYP) enzymes. The 25-hydroxylation is by cholesterol 25-hydroxylase (Ch25H). Enzymatic production of a 5α,6α-epoxide leads to the formation of the 3β,5α,6α-triol by cholesterol epoxide hydrolase (ChEH) (Newman et al. 2005). Non-enzymatic formation of the 7β-hydroxy and 7-keto moieties is by autoxidation. Adapted from Olkkonen et al. (2012).

Cholesterol is intermittently entwined into the phospholipid bilayer, often esterified to fatty acids such as PUFAs to form cholesteryl esters (Brown et al. 1996). It strengthens phospholipid packing to both reduce membrane permeability and maintain fluidity during temperature fluctuations (Bhattacharya & Haldar 2000; Cooper 1978). The addition of polar regions (e.g. alcohols, ketones) leads to conformational changes; ring-oxidised COPs attain a tilted orientation between phospholipids, disordering membrane structure, while tail-oxidised COPs retain similar membrane orientation to cholesterol under high surface pressure (Kulig et al. 2015; Olkkonen & Hynynen 2009). Cholesterol also circulates bound to lipoproteins and is a precursor for vitamin D and steroid hormone synthesis (Miller 2013) and is a component of bile and derives bile acids (Javitt 1994). Both cholesterol and its oxidised derivatives are involved in cell signalling pathways including cell proliferation and differentiation, inflammation and immunity, and cell survival/apoptosis (Baranowski 2008; Bensinger et al. 2008; Huang et al. 2016; Lordan et al. 2009; Olkkonen et al. 2012; Sabuncuoğlu & Öztaş 2014; Vurusaner et al. 2016).
Bioactive endogenous COPs exert many of their cellular effects as agonistic ligands of the liver X receptor (LXR), where monooxidation of the side chain is a prerequisite for high affinity binding. The principal ligands require a 22-, 24- or 27-hydroxyl group, but 25-HC and, to a lesser extent, 7α-HC, may also act upon LXR (Fu et al. 2001; Janowski et al. 1999; Svensson et al. 2003; Trousson et al. 2009). Autoxidative COPs have been associated with cytotoxicity and apoptosis induction, which may occur independent of LXR or by LXR hyperactivation (Choe et al. 2007; Lordan et al. 2009; Nury et al. 2013; Trousson et al. 2009; Vurusaner et al. 2016; Wente et al. 2006; Zhang et al. 2014). The metabolic, tail-oxidised COPs (24-HC, 25-HC and 27-HC) have been implicated in the induction of adaptive cell protection in response to oxidative stress, such as that induced by ionising radiation exposures, which may also include activation of cell death pathways (Gamba et al. 2015; Noguchi et al. 2014; Okabe et al. 2014). The cholesterol precursor, lathosterol, is positively correlated with cholesterol biosynthesis (Figure 1.10), which can therefore indicate cholesterol availability for COP formation (Duane 1995; Olkkonen et al. 2017).

![Figure 1.10: Cholesterol biosynthesis from lathosterol.](image)

**Figure 1.10: Cholesterol biosynthesis from lathosterol.** Lathosterol is desaturated by hydrogen abstraction from C5 and C6, catalysed by lathosterol 5-desaturase, to form a C5 double bond. The intermediate, 7-dehydrocholesterol, is reduced across C7 and C8 by hydrogen addition, catalysed by 7-dehydrocholesterol reductase, to remove the C8 double bond and form cholesterol.

### 1.3.3 Dose-response

The amount of energy deposited within the body or tissue, depicted by the exposure dose, dictates the likely onset of biological effects concerning ionising irradiation (Medalia 2003). Many factors govern the exposure dose, including the source strength (activity), distance and shielding; the exposure time; the energies of individual emissions; the type of radiation (e.g. alpha, beta, gamma); the form of the material (e.g. powder, solid metal); and for internal contamination, the biokinetics and biodistribution of the radionuclide within the body (Medalia 2003). For the average person, natural background ionising radiation constitutes a dose of about
0.01 milligray (mGy)/day gamma-radiation equivalent (millisievert, mSv; Section 1.1.1, Table 1.2). From a biological standpoint, acute exposures above 150 mSv is considered high dose irradiation, while low dose irradiation lies between natural background and acute high dose levels (Bonner 2003; Desouky et al. 2015).

Clinical expression after ionising irradiation can manifest as stochastic (random) effects, such as neoplasia or heritable mutation, or deterministic effects, including the signs and symptoms of acute radiation syndrome (ARS) or radiation sickness (Boreham et al. 2006; Cardis et al. 2005; ICRP 2007; Weinberg et al. 2001). While the severity of deterministic effects is dose-dependent, it is the probability of incurring a stochastic effect, rather than the severity, that increases with dose (ICRP 2007). Mild deterministic effects may be observed after 0.5 Sv, haematopoietic dysfunction after 1.5 Sv, haematopoietic syndrome progression and death without supportive care after 4 Sv, gastrointestinal syndrome onset after 6 Sv, and neurovascular syndrome onset after 15 Sv with imminent death (Garau et al. 2011). Low and high dose stochastic effects are currently accepted to follow the linear nonthreshold (LNT) model, which suggests proportionality between dose and long term stochastic effect, such that the potential harm per absorbed dose from such effects is considered equal, independent of dose (Bonner 2003; Tubiana et al. 2009). However, this model does not consider biological defence and signalling effects, which are described by two prevailing alternative hypotheses (Desouky et al. 2015).

The first is the adaptive-response model, where low-dose irradiation prepares the cell for subsequent high dose exposures, reducing damage anticipated by the LNT model. The adaptive response, or radiation hormesis, coincides with cell adaptation to oxidative stress (Section 1.3.1), accompanying upregulation of cellular defence mechanisms, inclusive of ROS scavengers/antioxidants, DNA repair pathways and cell death or senescence to prevent replication of mutagenic effects (Azzam et al. 2012; Feinendegen et al. 2004; Tubiana et al. 2009). These effects may be observed after a delay of several hours after a low dose exposure and may offer some protection for hours or months after exposure (Tubiana et al. 2009). The adaptive response is most active between 1-100 mGy gamma-irradiation at a dose rate of 0.01-1 Gy/minute (min), which progresses increasingly via apoptosis after 200 mGy, although hormesis can be elicited by up to 500 mGy exposures (Bonner 2003; Desouky et al. 2015; Feinendegen et al. 2004; Mitchel 2010; Tubiana et al. 2009).

The second model is the bystander-effect model, which proposes that cells irradiated to low
doses signal their distress to nearby cells, increasing damage predicted by the LNT model. Irradiated cells may provoke damage to bystander cells via intercellular signalling mechanisms (e.g. gap junctions) or secreted diffusible factors that are responsible for indirect effects (e.g. ROS, cytokines) (Desouky et al. 2015). However, contrary to this classical bystander model, cells irradiated to those doses required to initiate adaptive responses may also confer protection to bystander cells (Tubiana et al. 2009). The induction of both the adaptive response and bystander models may be dependent upon the LET of the radiation, where the potential for more severe, localised damage from higher LET particles may prevent such responses (Anzenberg et al. 2008; Dimova et al. 2008). However, despite such observations to the contrary, these effects have also been perceived for high LET radiations (Marples & Skov 1996).

### 1.3.3.1 Biodosimetric markers

To determine an individual’s risk for developing an ARS or stochastic effect, the importance of absorbed dose delivered during whole body or partial body irradiation is apparent (Section 1.3.3). A useful biodosimetric marker must produce a measurable and reproducible change in concentration from its normal (baseline) range that is dependent on the exposure dose. The change must also persist long enough post-exposure to be detected at the time of analysis. Biomarkers that have shown potential as biodosimetric indicators for radiation injury triage include proteins (e.g. cytokines, γ-H2AX), peripheral blood cell counts, chromosomal aberrations, ribonucleic acids (RNAs), and metabolites (Guipaud & Benderitter 2009; Rana et al. 2010; Singh et al. 2016; Zeegers et al. 2017). Such studies have measured the dose-response of these markers from several hours up to days or even weeks post-irradiation. The severity and localisation of damage can also be ascertained by certain markers. In cases of accidental gamma-radiation exposure, plasma concentrations of cytokine Flt3 ligand was used to indicate haematopoietic integrity, citrulline to assess gastrointestinal damage, and various COPs to monitor damage to hepatic (7α-HC), cerebral (24-HC), pulmonary and cardiovascular (27-HC) tissues (Bertho et al. 2009; Bertho et al. 2008). One case (Bertho et al. 2009) involved an estimated dose of 1 to 2.3 Gy after numerous intermittent low dose rate exposures to an $^{192}\text{Ir}$ source, while another case (Bertho et al. 2008) involved an estimated dose of 4.2 to 4.8 Gy after a single high dose rate exposure to a $^{60}\text{Co}$ source. In each case, several weeks had passed between the time of initial exposure and hospital
admission for radiation injury assessment. Certain biomarkers were detected within their normal range due to differences in the dose, dose rates and delayed biological assessment, demonstrating a lack of initial damage or recovery of such tissues post-exposure. However, detection of changes to the COP profile in each diverse case suggested COPs to respond to varied doses and dose rates, as well as persist for extended periods (at least several weeks) post-irradiation. Although the exact response may vary over this time, COPs have demonstrated potential as a candidate for routine radiation injury triage and dose estimation.

While providing an indication of radiation injury, biomarkers such as COPs may also serve to incriminate a suspect in radiological crime. The perpetrators of such crime may be exposed to radiation during handling of the radioactive source container, where the dose received may be significant enough to cause radiation sickness, or it may be minor. For this purpose, a radiation biomarker should indicate not only radiation injury, but also exposure where injury has not occurred. Such is the potential for COPs that originate from the cellular adaptation to radiation exposure (discussed in Section 1.3.2.2.1).

The physiological roles and mechanisms of action for COPs have been investigated by comparison of animal or cell models before and after treatment with various COPs (Cheema et al. 2014; Lordan et al. 2009; Trousson et al. 2009; Vurusaner et al. 2016; Wente et al. 2006). Such experiments with exogenous COPs have implicated roles for these compounds after gamma-irradiation in human cells (Hyun et al. 2002). However, studies of COP formation in biological systems after gamma-irradiation have so far been limited, such that they have not examined endogenous COP formation and/or correlative effects between multiple doses. More comprehensive biodosimetry studies have excluded COPs from analyses, focusing on fatty/amino acid, protein or common urinary markers (Kwon et al. 2014; Menon et al. 2016; Tyburski et al. 2008). An evaluation of endogenous COPs after gamma-irradiation of human cells is therefore necessary, which would enable the potential roles for COPs in biodosimetry and radiation-induced effects to be determined. Exposure of cells to doses that are nonlethal or sufficient to cause an ARS ($\leq 10$-$15$ Gy) is required in order to establish any biodosimetric relationship for practical use.
1.4 Extraction and analysis of oxysterols

COPs may act as tissue-specific, composite indicators of ionising radiation-induced cellular oxidative damage, as well as physiologically-relevant metabolic alterations, in a dose-dependent manner (Sections 1.3.2.2 and 1.3.3.1). The methodologies available for the sample workup of COPs vary in their intricacies, but generally include (1) tissue homogenisation and COP extraction in a liquid-liquid solvent system; (2) alkaline hydrolysis (saponification) to liberate fatty acids from the parent sterol and purification of target compounds by a solid-phase approach; (3) derivatisation of analytes, as applicable for the analysis method; and (4) chromatographic separation of analytes and detection/quantification via a mass spectrometric approach.

1.4.1 Liquid-liquid extraction

Cellular lipids and non-lipids, such as proteins, are closely associated with each other through interactions including hydrogen bonding and hydrophobic, dipolar and dispersion forces. Isolation of lipids therefore requires that these interactions be disrupted so that lipids and non-lipids can be separated (Georgiou et al. 2014). Due to its low cost and simplicity, a liquid-liquid extraction (LLE) system is most frequently employed for this purpose, generally consisting of a mixture of organic solvents of differing polarity that are able to form an immiscible and thus biphasic system (Georgiou et al. 2014; Mubarak et al. 2015). Lipids, comprising mostly nonpolar groups, selectively partition into nonpolar solvents that disrupt hydrophobic and other nonpolar interactions. Inclusion of a polar solvent dissolves polar non-lipids and assists in the penetration of the cell by way of disturbing hydrogen bonding and other polar interactions, increasing lipid availability to the nonpolar solvent (Li et al. 2014). A mechanical homogenisation (e.g. blender, ultrasonic probe) can be applied in conjunction with such solvent systems for the digestion of tissue samples. Non-soluble protein contaminants precipitate at the liquid-liquid interface.

One of the first and most commonly adapted lipid extraction methods using such a solvent system was devised by Folch et al. (1957) for various tissue samples. The original Folch method utilised a mixture of chloroform/methanol (2:1 v/v) for sample homogenisation to a final volume 20 times that of the sample. Considering water endogenous to the sample, proportions of chloroform/methanol/water were kept constant at 8:4:3 (v/v/v), separating an
upper aqueous phase and lower organic phase predominated by methanol/water (polar) and chloroform (nonpolar), respectively. Losses of polar lipids (e.g. COPs) into the aqueous layer can occur where improper ratios of solvent to water are used (Christie 1993). This method is considered the most efficient and reliable for complete isolation of lipids from various tissues, hence why it remains so widely regarded (Georgiou et al. 2014).

A modification of the Folch method by Bligh and Dyer (1959) is the second most commonly used and adapted LLE protocol. The Bligh/Dyer method was developed for extraction of total lipids from fish tissue containing about 80 % water and 1 % lipid, with reduced solvent volumes. Homogenisation is carried out in chloroform/methanol (1:2 v/v) three times the sample volume, after which, chloroform/water (1:1 v/v) is added to achieve phase separation. The upper aqueous (methanol/water) phase containing non-lipids is removed to isolate lipids into the lower organic (chloroform) phase. Adaptation of this method to other sample types is possible, although it is important that proportions of chloroform/methanol/water be maintained at 1:2:0.8 and 2:2:1.8 (v/v/v) before and after phase separation, respectively, to prevent lipid losses. Thus, for samples not containing 80 % water, volumes of chloroform/methanol and/or water must be adjusted accordingly. Bligh and Dyer (1959) found that lipid yields with this method did not differ significantly from that of the Folch method, with the main advantages being economical through convenience of solvent volumes relative to sample (Christie 1993).

The original Folch and Bligh/Dyer methods were evaluated by Iverson et al. (2001) for their ability to extract lipids from marine tissues. One important difference identified between these two methods was their ability to handle samples with high lipid content. While lipid yields did not differ for samples comprising below 2 % w/w lipid, above this threshold, the Bligh/Dyer method significantly underestimated lipid content; this was by as much as 50 % in the highest lipid samples (26.6 % w/w lipid). It was suggested this might result from lipid solubility limits being reached in the organic phase when solvent volumes are decreased and lipid content increased, since reduced solvent/sample (7.5:1 v/v) Folch extractions also underestimated lipid content exceeding 3 % w/w (Iverson et al. 2001).

Solvent toxicity is another drawback to these methods, and as such, alternative LLE systems have been developed using less toxic solvents, such as hexane and isopropanol (Hara & Radin 1978). Many such solvent systems have been evaluated on various sample types by numerous studies in comparison to the Folch and/or Bligh/Dyer methods or similar (Axelsson & Gentili 2014; Balasubramanian et al. 2013; D'Oca et al. 2011; de Morais et al. 2010; dos Santos et al. 2013).
Each of these studies that used a form of the Folch method identified the chloroform/methanol (2:1 v/v) system as being superior to, or otherwise on par with (Axelsson & Gentili 2014), other solvent systems for overall lipid recovery. A mixture of hexane/isopropanol (3:2 v/v) was better at extracting nonpolar lipids and methanol/tert-butyl methyl ether (1:1.25 v/v) was better for lactosyl ceramides (Reis et al. 2013). The Bligh/Dyer method did not stand out from other extraction systems (de Morais et al. 2010; Li et al. 2014).

1.4.2 Purification of oxysterols

Purification of COPs from co-extracted, organic-soluble compounds is necessary to prevent interference during later chromatography. The organic partition following solvent extraction will contain compounds such as lipid-soluble vitamins, acylglycerols, phospholipids, fatty acids, as well as free and conjugated cholesterol and COPs, commonly esterified to fatty acids (Brown et al. 1996; Georgiou et al. 2014; Yin et al. 2002). Thus, in addition to the selective isolation of target compounds and effective removal of contaminants, it is necessary for a purification procedure to hydrolyse lipid esters in order to release free analytes.

1.4.2.1 Alkaline hydrolysis (saponification)

Saponification involves the incubation of samples with a methanolic or ethanolic solution of a strong base, most frequently potassium hydroxide (KOH). The lipids are solubilised in the alcohol solvent while the hydroxyl ions of the base mediate the nucleophilic attack of the ester carbonyl group (C=O), separating the fatty acid carboxylic acid from the parent lipid alcohol (Hilal 2006). In a comparison of methanolic and ethanolic saponifications, ethanol use resulted in higher and more consistent oxysterol recoveries (Uphayasekera et al. 2004). This technique can also be performed directly, bypassing the initial solvent extraction (Lee et al. 2008b). Direct saponification has the advantage of reducing solvent consumption while enhancing reproducibility, but can also lead to problematic emulsions and chromatogram interferences (Dionisi et al. 1998; Georgiou & Kapnissi-Christodoulou 2013).

Generation of artefacts from the in vitro degradation or autoxidation of analytes is a common problem associated with saponification. This has been strongly correlated to incubation temperature and the strength of alkaline (Busch & King 2010; Park et al. 1996; Uphayasekera et
al. 2004). These studies demonstrated 7-KC to be especially labile under thermal and alkaline conditions, and thus its dehydration to 3,5-cholestadien-7-one can be used to monitor artefact production during saponification. Use of ‘cold’ saponifications at room temperature with a mild alkaline (1 M KOH) are known to minimise artefact generation (Busch & King 2010; Park et al. 1996). Regardless, hot saponifications (e.g. 37 °C) still find favour as they can be completed in as little as 30 minutes (Lee et al. 2008b), while cold saponifications require 18-22 hours incubation time (Busch & King 2010; Georgiou & Kapnissi-Christodoulou 2013; Park et al. 1996; Ubhayasekera et al. 2004). Direct saponifications have also been shown to reduce artefact production (Dionisi et al. 1998). Transesterification is an alternative to saponification that uses milder conditions to reduce artefact production at the potential compromise of COP recoveries (Ubhayasekera et al. 2004).

1.4.2.2 Solid-phase extraction

Once esterified lipids have been liberated, sample clean up then proceeds via solid-phase extraction (SPE). SPE works by passing a liquid sample (mobile phase) through a column containing a solid sorbent (stationary phase). Analytes of interest are adsorbed by the sorbent based on their chemical attributes (e.g. hydrophobicity, polarity, charge), which are then exploited to selectively elute chemically diverse compounds by the sequential use of various solvents. The choice of sorbent and solvents therefore varies depending on the analytes to be isolated.

The procedure by Lee et al. (2008b) was adapted from previous methods by the same group (Jenner et al. 2007; Lee et al. 2006; Lee et al. 2004). This method utilised a mixed-mode, reversed-phase / strong anion exchange resin suitable for the stepwise purification of COPs and F₂-isoprostanes. In addition to hydrophobic (reversed-phase) and strong anion exchange groups, the mixed anion exchange (MAX) sorbent includes a hydrophilic region (Figure 1.11).
Figure 1.11: Structure of a mixed anion exchange (MAX) sorbent for oxysterol and F$_2$-isoprostane purification. The sorbent consists of a copolymer of hydrophilic N-divinylpyrrolidone for retention of polar compounds and hydrophobic divinylbenzene for reversed-phase retention of nonpolar/neutral compounds (oxysterols). The divinylbenzene is functionalised with a quaternary ammonium cation that is a strong anion exchanger ($pK_a > 18$) and is thus always charged independent of pH for retention of deprotonated weak acids (F$_2$-isoprostanes). Adapted from Brousmiche et al. (2008) and Waters Oasis sample extraction products website (www.waters.com).

It is important to consider the acid dissociation constant ($pK_a$) of analyte and sorbent functional groups, which dictates the protonation state based on pH; deprotonation occurs at a pH greater than the $pK_a$, rendering acids charged (anionic) and bases neutral, while a pH less than the $pK_a$ charges the base (cationic) and neutralises the acid due to protonation. The quaternary ammonium ($NR_4^+$) strong anion exchanger ($pK_a > 18$) is impartial to pH (Brousmiche et al. 2008). Similarly, COP hydroxyls ($pK_a \approx 18$) are always neutral, and thus COPs are mostly retained by the reversed-phase copolymer, although the slight polarity conferred by their hydroxyl groups may also allow them to interact with the hydrophilic region. F$_2$-isoprostanes are weakly acidic by convention of their carboxylic acid ($pK_a \approx 5$), which is deprotonated at elevated pH to enable selectivity for the anion exchanger (Janicka et al. 2010).

The pH of samples and solutions that contact the sorbent is therefore essential to ensure the appropriate protonation states for retention, where applicable. Samples are therefore pretreated with suitable pH buffer for this purpose, and similarly, the sorbent is also preconditioned with a solution of equivalent solvent/ionic strength and pH to activate the bonded phase and any functional groups (Janicka et al. 2010). The sample is loaded prior to stepwise addition of various solvents to allow for the elution of contaminants and analytes into separate fractions.
1.4.3 Derivatisation

Derivatisation is a process by which functional groups are altered in order to render a compound more amenable to analysis by a specific technique (Bowden et al. 2009). For COPs analysis by gas chromatography (GC) (Section 1.4.4), derivatisation is carried out to reduce molecule polarity, improving volatility and thermal stability of otherwise non-volatile and/or thermolabile analytes. Thermal lability and low volatility are, in general, properties consistent with large molecules containing polar groups with an active hydrogen, which participate in strong intermolecular bonding via hydrogen bonds. Additionally, these groups are prone to adsorption to active surfaces within the GC column, hindering or preventing their migration (Orata 2012). Carbonyl groups without active hydrogens, such as ketones, typically do not inhibit analysis by GC (Bowden et al. 2009). The C5 double bond stabilises 7-KC to prevent enolisation (Busch & King 2009). Therefore, in the analysis of COPs, hydroxyl groups are the targets of derivatisation reactions.

Silylation is the most frequently applied method to eliminate the functionality of hydroxyl groups. Common functional groups most susceptible to silylation proceed in order of alcohol > phenol > carboxylic acid > amine > amide; stronger reaction conditions are required to derivatise the less receptive moieties (Knapp 1979). Water is another prime target for silylation, thus it is important that derivatisation is carried out in the complete absence of moisture, as well as alcoholic solvent. The most common reactions involve the replacement of the active hydrogen with a trimethylsilyl (TMS) group via a bimolecular (second order) nucleophilic substitution (SN$_2$) reaction (Figure 1.12). The resultant TMS ethers cannot hydrogen bond with each other and are less polar than their former alcohols, increasing volatility and thermostability to improve chromatographic resolution and peak shapes (Guardiola et al. 2004).
Figure 1.12: Second order nucleophilic substitution (SN₂) reaction for the silylation of hydroxyl groups with N,O-bistrimethylsilyl trifluoroacetamide (BSTFA) and trimethyl-chlorosilane (TMCS). The oxygen atom of the alcoholic substrate acts as a nucleophile in the reaction with a trimethylsilyl (TMS) group. At the transition state (rate-limiting step) a partial covalent bond is formed as an electron is transferred from the oxygen atom to the silicon atom and from the silicon atom to the leaving group (X). As the oxygen-silicon covalent bond is fully formed, a hydrogen cation is lost from the alcohol, liberating a leaving group anion that ionically bonds with the hydrogen cation. Adapted from Knapp (1979) and Supelco (1997).

Common reagents for silylation, in order of silyl donor strength, include N-trimethylsilylimidazole (TMSI), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), N,O-bistrimethylsilylacetamide (BSA), N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) (Little 1999). Reagent should be applied in excess of the stoichiometric requirement for a 1:1 molar ratio of TMS donor to active hydrogen; about 2:1 molar ratio is recommended (Supelco 1997), or greater for difficult derivatisations. Donor strength is enhanced through the use of reagent blends; BSA/TMSI/TMCS (3:3:2 v/v/v) is a strong donor, while BSTFA/TMCS (99:1 v/v) is weaker, but very common (Guardiola et al. 2004). The TMCS in such blends acts as a silylation catalyst, which increases the reactivity of other reagents. As such, it is possible to apply BSTFA with up to 20 % TMCS to enhance derivatisation power (Supelco 1997). The byproduct of TMCS silylation reactions, hydrochloric acid, can be removed by the addition of base catalyst, pyridine, which can further assist derivatisation of difficult groups when used as a solvent (Bowden et al. 2009; Guardiola et al. 2004; Little 1999).

Steric hindrance of functional groups can limit silylation potential. For this reason, primary alcohols are more easily silylated than are secondary or tertiary alcohols, the latter being most difficult (Knapp 1979). The 5α-hydroxyl of 3β,5α,6β-cholestantriol (3β,5α,6β-CT) and 25-
hydroxyl of 25-HC are known sterically hindered groups; partial derivatisation to their respective bis-TMS and mono-TMS ethers are often observed under mild reaction conditions (Brooks et al. 1973; Busch et al. 2011; Lai et al. 1995; Missler et al. 1985). Silylation of CT to the tris-TMS ether is especially difficult; harsh reaction conditions often with lengthy incubation periods (beyond 24 hrs) are usually required for complete silylation of CT (Brooks et al. 1973; Park & Addis 1989). Thus, it is common to find only the bis-TMS ether of CT acquired post-derivatisation; this is advocated as a means to avoid artefacts associated with sterol decomposition under the harsher conditions required to obtain the tris-TMS ether (Guardiola et al. 2004; Little 1999).

Dehydration of 25-HC at the 25-hydroxyl to desmosterol, or of 7-KC at the 3-hydroxyl to cholesta-3,5-dien-7-one, are common artefacts with several potential causes. Shan et al. (2003) suggested their desmosterol artefacts could reflect the silylation conditions applied, or problems at the GC injector liner or mode of injection (split/splitless). Dehydration of 7-KC is often associated with thermal decomposition, although heating during the derivatisation process itself is not thought to contribute since the presence of silylation reagent acts as a protectant (Busch & King 2009). Thus, it is possible that incomplete derivatisation of this compound, such as from the use of suboptimal conditions or presence of moisture, may prevent heat protection during GC as the thermostable TMS ether. Other factors that may impact silylation artefacts include storage conditions and variability between reagent batches (Busch et al. 2011; Guardiola et al. 2004).

1.4.4 Gas chromatography coupled mass spectrometry

Capillary chromatography (Tan & Quanci 1985) is the standard approach for discrete separation of chemically similar compounds. Gas chromatography coupled to mass spectrometry (GC-MS; Figure 1.13) is considered the ‘gold standard’ for COPs analysis (Griffiths et al. 2016). For each isolated analyte that elutes from the GC, the MS generates predictable structural ions and amplifies their signals, improving sensitivity and providing molecular mass, structural and quantitative information pertaining to each analyte (Glish & Vachet 2003). While each technique alone is unreliable for analyte identification owing to the potential for coelution, isomers and matrix interferents (Lei et al. 2011), complementary use of chromatographic retention and mass spectral data enable highly accurate analyte identification and quantification.
Figure 1.13: Components of a gas chromatograph (GC) and mass spectrometer (MS). The sample is introduced by syringe injection into the GC injection port, where analytes are volatised and forced through a thin fused-silica capillary column by a gaseous mobile phase. Separation of analytes is by their volatility as the oven ramps the temperature and affinity to a stationary polymer (sorbent). As analytes elute from the GC, they are ionised within the MS ion source by an electron beam, then pass into a mass analyser (e.g. quadrupole), where ion fragments are filtered by mass-to-charge ratio (m/z) (e.g. within an alternating and direct current (AC/DC) field). Detection signal is amplified by an electron multiplier and recorded by retention time on a chromatogram and by m/z on a mass spectrum.

Alternatively, COPs may be analysed by liquid chromatography (LC). The major advantage of LC studies is the potential to bypass time-consuming and often difficult derivatisation techniques required to permit analysis by GC (Section 1.4.3), as well as the possibility for simplified sample preparation (AB Sciex 2010). Furthermore, LC studies can be used to measure conjugated COPs without the need to remove these groups (Griffiths et al. 2016). However, LC-MS is less sensitive and chromatograms less resolved than GC-MS, often necessitating derivatisation (e.g. to Girard P hydrazones or dimethylglycinates) to achieve sufficient sensitivity and resolution by enhancing polarity and MS ionisation efficiency (Jiang et al. 2011; Karu et al. 2007; Meljon et al. 2012; Narayanaswamy et al. 2015). Tandem MS is also required in order to match the sensitivity and selectivity of ionisation modes compatible only with GC (Krone et al. 2010; Shan et al. 2003). Further, pre-existing mass spectral libraries are available to GC-MS ionisation modes for identifying unknown compounds (Lei et al. 2011; Van Bramer 1998).
1.4.4.1 Mobile and stationary phase selection

The mobile phase (carrier gas), commonly helium, hydrogen, or nitrogen, is used to passage vaporised compounds through the GC system (Guardiola et al. 2004). Helium is generally the carrier gas of choice as it is inert and can flow optimally at linear velocities to enable faster GC than nitrogen, without sacrificing peak resolution (Munoz-Guerra et al. 2011; Sciarrone et al. 2015). While hydrogen is less viscous and thus capable of faster GC than helium, it is a combustible and reactive gas that can protonate analytes, yielding some problematic ion variations when detection methods such as MS are applied (Munoz-Guerra et al. 2011; Nnaji et al. 2015). Use of hydrogen also requires a more efficient vacuum system to prevent losses in sensitivity (Munoz-Guerra et al. 2011).

COPs and their precursors can be separated on a wide range of liquid stationary phases, including nonpolar 100%-polydimethylsiloxane (PDMS), low-polar 5%-phenyl-PDMS or medium-polar 35-65%-phenyl-PDMS or 14%-cyanopropylphenyl-PDMS (Guardiola et al. 2004; Rodriguez-Estrada et al. 2002). Park and Addis (1985) indicated that increasing column polarity worsened COP resolution. As sorbent polarity increases, so too does thermolability, such that low- and medium-polar sorbents require thermostable design in order to reduce column degradation (bleed) at high operating temperatures. Thermostable polar columns offer reduced bleed and superior resolution to traditional nonpolar columns, with low-polar sorbents regarded to better resolve COPs than medium-polar sorbents (Guardiola et al. 2004; Rodriguez-Estrada et al. 2002).

1.4.4.2 Ion source

Ionisation of analyte molecules is carried out in the ion source of the mass spectrometer. The ionisation can generate a molecular ion without changing the composition or mass of the intact analyte, or can cleave the analyte molecule into smaller fragment ions (Van Bramer 1998). For analysis of COPs, the ion source usually comprises electron ionisation (EI), as detailed by Ashcroft (1997) and Gross (2011). In summary, the EI source consists of a filament that is electrically heated to produce free electrons by thermionic emission, which are concentrated into a beam by attraction toward a positive trap electrode. Analytes are ionised upon passing through the electron beam and a repeller electrode accelerates the ions into the mass analyser (Section 1.4.4.3). The electron beam of an EI source has sufficient energy, typically 70 eV, to
displace a valence shell electron, fragmenting analytes predominately into positive ion radicals. Typical fragmentation ions of common COPs are indicated in Table 1.3.

Table 1.3: Five most abundant characteristic ions (m/z) and relative abundances (%) for common oxysterols and precursors generated with a 70 eV electron ionisation source.

<table>
<thead>
<tr>
<th>Sterol (molecular ion)</th>
<th>Characteristic ions (relative abundance)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (458)</td>
<td>458 (27), 368b (59), 353c (32), 329d (77), 129e (100)</td>
</tr>
<tr>
<td>Lathosterol (458)</td>
<td>458 (89), 443f (24), 353c (19), 255g (100), 213h (41)</td>
</tr>
<tr>
<td>7-KC (472)</td>
<td>472 (100), 382b (18), 367c (53), 161d (47), 129e (76)</td>
</tr>
<tr>
<td>7α/β-HC (546)</td>
<td>546 (1), 456b (100), 441c (1), 366d (3), 129e (11)</td>
</tr>
<tr>
<td>24-HC (546)</td>
<td>413f (44), 323g (12), 159h (44), 145i (100), 129e (54)</td>
</tr>
<tr>
<td>25-HC (546)</td>
<td>546 (2), 456b (4), 327h (6), 271i (10), 131j (100)</td>
</tr>
<tr>
<td>27-HC (546)</td>
<td>546 (14), 456b (41), 441c (16), 417d (42), 129e (100)</td>
</tr>
<tr>
<td>19-HC (546)</td>
<td>531e (1), 456b (5), 366d (53), 353f (100), 145i (48)</td>
</tr>
</tbody>
</table>

a Fragment ion structures from Pizzoferrato et al. (1993)
b [M-TMS.OH]+ (M-90) 
c [M-CH3+TMS.OH]+ (M-105) 
d [M-C1,C3+TMS.O]+ (M-129) 
e [M-(M-C1,C3+TMS.O)]+ (M-(M-129)) 
f [M-CH3]+ (M-15) 
g [M-C20+TMS.OH]+ (M-203) 
h [M-C20+CH3OH+TMS.OH]+ (M-245) 
i [M-2TMS.OH]+ (M-180) 
j [M-C25,C27+TMS.OH]+ (M-133) 
k [M-C25,C27+2TMS.OH]+ (M-223) 
l [M-(M-C24,C25+TMS.O)]+ (M-(M-145)) 
m [M-C1,C3+2TMS.O(H)]+ (M-219) 
o [M-(M-C24,C25+TMS.O)]+ (M-(M-131))

While fragmentation patterns are useful for inferring atomic structure, interpretation of analyte molecular mass relies on the presence of the molecular ion (Van Bramer 1998). As a hard ionisation technique, EI produces many fragment ions and accordingly, mass spectra are complex, with little or no molecular ion. The benefit of EI is its standardised 70 eV ionisation energy and subsequent reproducibility of mass spectra, which simplifies interpretation of complex spectra by enabling matching of unknowns to pre-existing spectral libraries (Lei et al. 2011; Van Bramer 1998).
1.4.4.3 Mass analyser

Ion motion and trajectory can be manipulated within an electric and/or magnetic field based on both mass and charge. Electromagnetic forces act more strongly upon ions with greater charge, with lighter ions being more amenable to such forces than heavier ions. The mass analyser exploits these properties to sort ions by mass-to-charge ratio ($m/z$) prior to detection. The energies utilised by the ion source are often not sufficient to liberate more than a single electron at a time, so that the $m/z$ often corresponds to the fragment molecular mass. The $m/z$ is measured as kilograms per Coulomb (kg/C) from the International System of Units (Glish & Vachet 2003).

The quadrupole mass filter is the most extensively applied mass analyser owing to its low cost relative to other analysers, ease of automation, low voltage requirement for ion acceleration from source to analyser, high transmission efficiency (i.e. number of ions produced relative to number of ions that reach the detector), modest vacuum requirements, compact size, and faster scan rate relative to magnet-containing analysers (Glish & Vachet 2003; Miller & Denton 1986; Van Bramer 1998). The quadrupole is a beam analyser that separates ions in space; ions generated in the ion source enter the analysing field of the mass analyser as a beam, only reaching the detector given an unobstructed trajectory (Glish & Vachet 2003; Miller & Denton 1986). Only a single $m/z$ is permitted to the detector at once, which necessitates rapid and continuous scanning through the $m/z$ range, causing a loss in sensitivity as compared to analysers that permit all ions to reach the detector via separation in time, such as time-of-flight or trapping analysers (Fitzgerald et al. 1997; Hoker et al. 2015; Van Bramer 1998).

The quadrupole mass filter separates ions based on their motion in a dynamic electric field, as per the Mathieu equation (Ruby 1996). The electric field is generated between four parallel cylindrical or hyperbolic electrode rods, arranged in crosswise pairs on the $X$-$Z$ and $Y$-$Z$ axes (Glish & Vachet 2003; Miller & Denton 1986). A direct current (DC) and radiofrequency (RF) field is connected to and governs the polarity of each electrode pair. The DC voltage applies a constant positive polarity to the $X$-$Z$ electrodes and negative polarity to the $Y$-$Z$ electrodes, which drive ions toward electrodes of opposite charge. The ions are pulled back toward centre by the alternating current (AC) of the RF field, which transiently switches the electrode polarities, causing the ions to spiral as they refocus on each plane (Miller & Denton 1986; Van Bramer 1998).
Lighter ions respond rapidly to the oscillating electric field, moving in larger spirals (waves) with greater amplitude than heavier ions, which are influenced little by the AC. Instead, the movement of heavier ions is dictated by the average electrode polarity as determined by the DC, which gradually drift off-centre. Ions below and above a mass threshold, governed by the DC and RF fields, collide with the electrodes due to excessive wave amplitude or excessive drift, respectively, becoming neutralised (Miller & Denton 1986; Van Bramer 1998). The quadrupole size and frequency of the RF are usually kept constant, as is the DC and RF voltage ratio, which controls peak resolution; it is the DC and RF voltage magnitudes that are altered to selectively scan m/z over a specified range (Glish & Vachet 2003). Improvements to sensitivity can be achieved by limiting the number of scans and thus undetected ions.

If operated in scanning mode, the mass analyser rapidly scans and permits entry of all ions (typically hundreds) into the detector over a predetermined m/z range. Scanning mode is useful for non-targeted novel compound discovery, or for determining the most characteristic/abundant ions for design of a more selective mode of operation (Krone et al. 2010). Selected ion monitoring (SIM) is used when analyte and characteristic ions are already known and scans only preselected, indicative ions (typically three per analyte) at the time of analyte chromatographic elution. This allows more rapid scanning between m/z, thus permitting a greater number of ions to reach the detector as compared to scanning mode. In addition to improved sensitivity, SIM mode operation yields cleaner chromatograms, since non-targeted compounds are not detected.

1.4.4.4 Mass spectrometric detection

The MS detector is responsible for capturing the ions, which have been sorted by the mass analyser, as an electronic signal that is proportional to the number of ions detected. There are numerous detectors for MS, their use depending largely on the application, as well as sensitivity and speed (Neetu et al. 2012). For most applications, the actual number of ions entering the detector region of the MS is not sufficient to produce a signal with high sensitivity. Many mass spectrometers therefore amplify the ion signal using an electron multiplier, commonly of a discrete-dynode or continuous-dynode format, which are useful for amplifying low ion currents, below $10^{-15}$ ampere (Neetu et al. 2012).

The discrete dynode electron multiplier consists of 15-18 dynodes composed of a metal oxide, while the continuous dynode electron multiplier consists of a curved glass tube or ‘horn’
imbued with lead to form a resistive conductive surface. These surfaces are highly electron emissive, which release electrons when charged particles (ions or other electrons) strike the surface atoms. Electrons released from the first dynode are sequentially transferred by a magnetic field onto subsequent discrete dynodes, or by voltage gradient along the continuous dynode tubing, which increase in positive potential to result in a cascade of secondary electron emissions (Neetu et al. 2012). The exponential amplification of electrons increases the number of electrons received by the final collection electrode, vastly improving sensitivity.

1.4.4.5 Qualitation and quantification by GC-MS

GC-MS offers two-dimensional data; each chromatographic peak of particular area represents the sum of signal intensities for all scanned mass spectral ions for the eluting compound at a particular retention time. This is the total ion chromatogram (TIC) and can include ions detected by scanning or SIM modes of operation (Section 1.4.4.3). TIC or SIM ions can also be filtered by m/z post-analysis to display peak areas for only a single target ion, which is termed the extracted ion chromatogram (EIC). Usually, three ions are monitored per analyte in SIM mode, a single quantifier ion and two additional qualifier ions. Qualitative specificity by GC-MS relies on the combination of retention time and mass spectral pattern unique to each analyte, while quantification is based on the relative peak area of the quantifier ion as compared to that of an internal standard.

The internal standard must be non-native to samples and physically/chemically analogous to the analyte species of interest to ensure similar extraction properties, stability, and chromatographic and mass spectral response. A known amount of internal standard is carried through the entire analytical procedure alongside sample analytes, which monitors any influence of sample preparation or matrix effects on the chromatography or recovery of analytes. Retention time variation can also be normalised to the internal standard, called the relative retention time. A metabolic COP, 19-HC (Figure 1.14), is often used as an internal standard for COP analyses, given its structural/biochemical similarities to other COPs and absence from biological specimens (Grau et al. 2001; Guardiola et al. 1995; Shentu et al. 2012; Yamaga et al. 2002). Variations introduced by the chromatography (e.g. injection volume) and analyte recovery are compensated for by the addition of a second standard, the recovery standard, just prior to derivatisation (Grau et al. 2001; Guardiola et al. 1995). The purpose of the recovery standard is to monitor the recovery of the internal standard, from which the recovery of analytes can be
inferred (Guardiola et al. 2004). The recovery standard does not need to exert the same chemical properties as analytes for the purpose of extraction, but does need to be resolvable under the same chromatographic conditions and amenable to the same derivatisation and detection method. The high-boiling triterpene, betulin, can be resolved as a TMS ether at similar temperature to COPs on a 5%-phenyl-PDMS-equivalent stationary phase, with detection by 70 eV EI-MS (Šiman et al. 2016), and is therefore suitable as a recovery standard for COPs analysis using the same techniques (Figure 1.14).

![Internal standard](image1.png) ![Recovery standard](image2.png)

**Figure 1.14: Structures of 19-hydroxycholesterol (internal standard) and betulin (recovery standard).** Adapted from Kim et al. (2016).

A calibration curve can be used to estimate the concentration of analyte when this is unknown, based on the linear response of a dilution series. Calibration curves are generated for EICs of each SIM quantifier ion relative to the internal standard, which is held at constant amount. The relative analyte response at known concentration is determined from the preparation of calibration samples within a matrix that closely mimics that of the test sample. For reliable quantification (and qualitation), analyte peaks must be distinguishable from the background or baseline chromatographic noise, attributed to column bleed and detector/electrical noise. Theoretical instrument sensitivity is generally accepted as the signal-to-noise ratio (S/N), where a limit of detection (LOD) is generally considered as three times the noise (S/N = 3) and a limit of quantification (LOQ) is equal to signals ten times the noise (S/N = 10) (Saadati et al. 2013; Shrivastava & Gupta 2011). In practice, LOD and LOQ should correspond to the lowest amount of an analyte that can be reliably distinguished from the noise (LOD) and quantified without compromising accuracy or precision (LOQ), such as due to deviations from linearity (Armbruster & Pry 2008).
1.4.5 Artefact avoidance

Artefacts are compounds acquired by preparative analytical processes that may or may not differ from analytes intrinsic to the original sample. Artefact formation can therefore overwhelm the endogenous COPs originally present in the sample matrix. Breakdown of COPs during saponification (Section 1.4.2.1) and derivatisation (Section 1.4.3) has been discussed. However, artefactual cholesterol autoxidation is the primary means by which COP yields are artificially increased, which has repercussions for both qualitative and quantitative analyses (Schroepfer Jr. 2000; Smith 1980; Smith 1981). Precautionary measures to reduce autoxidation include minimising or eliminating exposure to oxygen, light, metals, and heat during sample workup (Busch & King 2009). Common practice includes immediate flash freezing of samples upon collection without thawing until analysis and use of antioxidants added into the lipid extraction system (Liu et al. 2009).

Antioxidants include any molecule that mitigates oxidative damage by interfering with the generation of prooxidative species. They can do this in a variety of ways, for example, by scavenging free radicals, chelating metal ions that would catalyse free radical production (e.g. Fenton reaction), or by inhibiting prooxidant enzymes and activating antioxidant enzymes (Lü et al. 2010). Antioxidants with reduction potentials greater than that of cell components undergo preferential oxidation in place of oxidisable cell structures. The oxidised antioxidant radical is stabilised through radical-radical conjugation (Section 1.3.1), or can be regenerated after reduction in the presence of another antioxidant of greater reduction potential (Buettner 1993). These antioxidants are able to terminate free radical chain reactions (e.g. lipid peroxidation, Section 1.3.2.2), usually following the transfer of a single electron in the form of a hydrogen atom to the free radical, reducing it to a stable form (Yehye et al. 2015).

Butylated hydroxytoluene (BHT; Figure 1.15) is commonly applied to the sample workup of COPs. As a lipophilic antioxidant, it is typically dissolved in the methanol used for solvent extraction and is recovered in the organic partition along with the lipid fraction. Inclusion of BHT prevented artefactual COP formation in atherosclerotic plaques, while without BHT, Helmschrodt et al. (2013) found that potential autoxidation increased by three-fold. Further, it was found that 50 µg BHT per 1 mL (millilitre) sample was ideal, which is consistent with BHT use in studies of plasma and cell suspension samples (Pataj et al. 2016).
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Figure 1.15: Structure of butylated hydroxytoluene (BHT). BHT is a highly effective antioxidant on account of its di-tert-butyl groups located on its phenolic ring, which protonate lipid peroxides in order to terminate peroxidation chain reactions (Yehye et al. 2015).

1.5 Contribution of this research

Forensic investigation of a radiological crime may rely upon DNA identifications. Ionising irradiation of DNA evidence may impede PCR-based analyses, where degradation of the STRs has been the topic of prior investigation (Abbondante 2009; Goodwin 2013). These studies revealed potential for poor genotypes after exposure of DNA to high doses of gamma-radiation (> 1 kGy).

Oxidative reactions are the main source of biological damage due to gamma-irradiation, generated from water radiolysis and hyperproduction of mitochondrial ROS (Leach et al. 2001; Yamamori et al. 2012). This is understood to heighten mtDNA oxidative damage over nuDNA (Richter et al. 1988; Yakes & van Houten 1997). However, the impact of gamma-radiation upon mtDNA in relation to forensic STR markers, which have been isolated from the same samples after doses previously recognised to cause degradation of STR genotypes, is currently unknown.

The same oxidative processes that may lead to differential DNA damage may also be exploited for the generation of novel biodosimetric markers, such as COPs, after low doses of gamma-radiation (< 10 Gy). The potential of circulating metabolic COPs (7α-, 24-, 27-HC) has been demonstrated for estimating gamma-radiation exposure dose and sites of radiation injury after accidental exposures (Bertho et al. 2009; Bertho et al. 2008). This application could be extended to indicate likely perpetrators of a radiological crime. A microanalytical GC-MS approach could provide the sensitivity required to assay these and additional metabolic (25-HC)
and autoxidative (7\(\beta\)-HC, 7-KC) COPs, as a measure of the cellular response to biologically relevant low doses of gamma-radiation. While the function of COPs in the survival and detriment of the cell is well researched (Section 1.3.2.2.1), the potential of such biomarkers in biodosimetry and to elucidate cellular processes leading to gamma-radiation damage has not been investigated over multiple controlled doses (Section 1.3.3.1).

The principal aims of the research were to:

1. Develop procedures for the analysis of mtDNA degradation (Chapter 2);
2. Compare the degradation of nuDNA and mtDNA targets for forensic practicality after various high doses of gamma-radiation (Chapter 3);
3. Develop procedures for the quantification of COPs by GC-MS (Chapter 4); and,
4. Evaluate the generation of cellular COPs as dose-dependent metabolic and oxidative biomarkers after various low doses of gamma-radiation (Chapter 5).
Chapter 2: Development of methodology for the analysis of mitochondrial DNA degradation
2.1 Introduction

A real-time PCR procedure was developed by the Australian Centre for Ancient DNA (ACAD), with the intention to amplify multiplexed mtDNA targets of different size to assess mtDNA degradation (unpublished). Targets were therefore selected on their suitability for multiplexing, incorporating homologous primer and probe binding sites. Such sites were identified within the mtDNA coding region, but not the control region containing the HVRs. Specifically, targets were located in the 12S and 16S ribosomal RNA (rRNA) coding region. This approach, where each target was labelled with a different fluorophore, was not successful. The aim of this study was to adapt this procedure for singleplex reactions with SYBR Green. The final results are presented as a published manuscript (Section 2.2); however, additional developmental work was undertaken, which was not published (Section 2.3). The final developed assay was applied for examination of mtDNA degradation after gamma-irradiation (Chapter 3).
2.2 Published manuscript

2.2.1 Permissions and declaration

DECLARATION OF CO-AUTHORED PUBLICATION CHAPTER

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performed optimization study for a procedure to purify mtDNA standard, qPCR validation study, data analysis and prepared the manuscript.</td>
<td>60</td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Contributor is also a student at UC Y/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denise Higgins</td>
<td>Performed the target selection, primer design, and target specificity study, and reviewed the manuscript.</td>
<td>N</td>
</tr>
<tr>
<td>Shanen S. Tobe</td>
<td>Performed the target selection, primer design, and target specificity study, and reviewed the manuscript.</td>
<td>N</td>
</tr>
<tr>
<td>Jeremy Austin</td>
<td>Performed the target selection, primer design, and target specificity study, and reviewed the manuscript.</td>
<td>N</td>
</tr>
<tr>
<td>Andrew Wotherspoon</td>
<td>Provided advice and reviewed the manuscript.</td>
<td>N</td>
</tr>
<tr>
<td>Michelle E. Gahan</td>
<td>Provided advice and assisted with planning of the qPCR validation study, and reviewed the manuscript.</td>
<td>N</td>
</tr>
<tr>
<td>Dennis McNevin</td>
<td>Provided advice and assisted with planning of the qPCR validation study, and reviewed the manuscript.</td>
<td>N</td>
</tr>
</tbody>
</table>

Candidate’s Signature: [Signature]

Date: 05 March 2018

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate’s contribution to this work, and the nature of the contribution of each of the co-authors;

(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s): National Centre for Forensic Studies, Faculty of Science and Technology, University of Canberra

Date: 15 May 2018

<table>
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<tr>
<td>Denise Higgins</td>
<td>[Signature]</td>
<td>02 March 2018</td>
</tr>
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<td>Shanen S. Tobe</td>
<td>[Signature]</td>
<td>05 March 2018</td>
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<tr>
<td>Jeremy Austin</td>
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<td>Michelle E. Gahan</td>
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<tr>
<td>Dennis McNevin</td>
<td>[Signature]</td>
<td>02 March 2018</td>
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</table>

2.2.2 Singleplex quantitative real-time PCR for the assessment of human mitochondrial DNA quantity and quality

Corey Goodwin 1, Denice Higgins 2, Shanen S. Tobe 3,4, Jeremy Austin 2, Andrew Wotherspoon 5, Michelle E. Gahan 1, Dennis McNevin 1

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Abstract
Mitochondrial DNA (mtDNA) can provide a means for forensic identity testing when genotyping of nuclear DNA (nDNA) targets is not possible due to degradation or lack of template. For degraded samples, an indication of the quantity and quality of mtDNA is essential to allow selection of appropriately sized targets for hypervariable region (HVR) analysis, which may conserve sample and resources. Three human-specific mtDNA targets of increasing length (86, 190 and 452 base pairs) were amplified by singleplex quantitative real-time PCR (qPCR), capable of providing an index of mtDNA degradation from fragment length information. Quantification was achieved by preparation of a standard curve for each target, using a purified mtDNA standard containing all three targets of interest, which produced a linear, accurate and precise result from $1 \times 10^3$ to 10 copies. These novel assays demonstrated excellent sensitivity, specificity and reproducibility in line with the minimum information for qPCR experiments (MIQE) guidelines. Further, a separate inhibition control reaction was included to guide sample clean-up and ensure the validity of degradation assays. This protocol assists the selection and analysis of appropriately sized targets to maximize the chance of obtaining an informative result in downstream assays like sequencing.

Keywords Degradation · Mitochondrial DNA · Quantification · Quantitative real-time PCR · SYBR green

Introduction

The high copy number and maternal inheritance of mitochondrial DNA (mtDNA), as well as its extensive characterization in human populations worldwide, make it an extremely useful analysis tool for identification of degraded or ancient samples for population and evolutionary studies, as well as forensics in cases where nuclear DNA (nDNA) evidence is unavailable. Within the mtDNA control region (positions 16,024–16,569; 1–576 in the revised Cambridge Reference Sequence, rCRS), the hypervariable regions (HVRs) are common targets. These regions contain relatively high sequence variability, compared to the mtDNA coding region, and provide a means of forensic identity testing, as well as distinction between maternal lineages and geographic ancestry. For the low-template and/or degraded DNA often encountered within forensic and ancient samples, a reliable method of DNA quantification must assess both the quantity and quality of available mtDNA before proceeding with downstream and often expensive analyses, such as sequencing. An assessment of intact, amplifiable mtDNA quantity, a direct indicator of polymerase chain reaction (PCR) success, can be achieved by quantitative real-time PCR (qPCR).

Current validated procedures for quantification of mtDNA prior to HVR sequencing are based on a single mtDNA target [1, 2]. Alternatively, quantification of a mtDNA target may be combined with a nDNA target to provide an indication of mt DNA quality [3–6]. However, these assays do not offer
detailed mtDNA quality information relevant to common target lengths for HVR analysis, which is often achieved by fragmenting the control region into several amplicons that usually span around 150–400 base pairs (bp) [7–10]. While the quantity of amplifiable DNA is reduced for longer amplicons, which incur DNA damage lesions with greater frequency [11], this provides a means to assess DNA degradation by quantifying at least two mtDNA targets of different size [10, 12–14]. An index of DNA degradation of relevance to HVR analysis can then be determined from the quantity ratio of long versus short amplicons of similar lengths to common mtDNA targets.

This report describes a singleplex qPCR workflow to target three human mtDNA amplicons of different lengths, including a long (452 bp) and medium (190 bp) target of similar length to HVR amplicons, and a short (86 bp) target to represent mtDNA availability for massively-parallel sequencing enrichment. All targets were selected within highly conserved regions of the 12S ribosomal RNA (rRNA) and 16S rRNA sections of the mtDNA coding region to ensure homology across diverse human population groups and reduce the likelihood of primer mismatch from mutation. The minimum information for publication of qPCR experiments (MIQE) guidelines [15, 16] were applied in the assay design, development and validation to ensure high quality.

Methodology

DNA samples

DNA extracts were from single source human buccal swabs extracted with the ChargeSwitch Forensic DNA Purification Kit (Invitrogen, Carlsbad, USA) into 150 μL elution buffer, following the manufacturer’s protocol. Extracts were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen) with Qubit 2.0 Fluorometer (Invitrogen). Samples were stored at -20°C prior to use.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer (5'–3')</th>
<th>Reverse primer (5'–3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 bp</td>
<td>COGAAAYAGTACCCTCAAGGAT</td>
<td>TGAACCYATAGGCTGCAYCT</td>
</tr>
<tr>
<td>190 bp</td>
<td>TGCTAGGGTCTAAACTCCCTCAACA</td>
<td>GGGTCTTAGGAAGATGCCG</td>
</tr>
<tr>
<td>452 bp</td>
<td>ACAGCTCTTTTGGACACACTAGGA</td>
<td>ACAGTGTACTGCGGACGG</td>
</tr>
<tr>
<td>IPC</td>
<td>CAGGCCAGAGCGATACTT</td>
<td>CAGGGTAAATTGCCGAGCT</td>
</tr>
<tr>
<td>2119 bp standard</td>
<td>AGTCAATAGAGACCGCGCTGT</td>
<td>AGCAACCTTTAAATAGCAGCTG</td>
</tr>
<tr>
<td>IPC sequence (5'–3')</td>
<td>CAGGCCAGAGCGATACTTACATGCG</td>
<td>CAGGGTAAATTGCCGAGCT</td>
</tr>
</tbody>
</table>

1 bp = base pairs
(1 µL) MyTaq DNA Polymerase, 0.4 µM of each forward and reverse primer, and PCR grade water. Amplification was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA), with PCR cycling conditions comprising an initial denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min, then a final extension at 72 °C for 10 min. PCR clean-up was achieved with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol, and eluted into 30 µL elution buffer (10 mM Tris-Chloride, pH 8.5).

Purity of the mtDNA standard was determined from 2 µL amplified product based on absorbance ratios (duplicate measures) at 260/280 nm and 260/230 nm using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Waltham, USA), while DNA concentration (five replicate measures) was determined using a Qubit 2.0 Fluorometer with the Qubit dsDNA BR Assay Kit (Invitrogen). The stock concentration (ng/µL) was converted to copy number, CN (Eq. 1).

\[
CN = \frac{6.022 \times 10^{23} \text{ copies/mol} \times \text{amplicon mass (g)}}{\text{amplicon length (bp)} \times 650 \text{ g/mol/bp}}
\]  

(1)

Quantitative real-time PCR

Singleplex qPCR assays for each target and IPC were optimized for use with FastStart SYBR Green Master (Roche, Basel, Switzerland) on a 7900 Real-Time PCR System (Applied Biosystems). Thermal cycling was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 10 s, then a final extension at 60 °C for 1 min. The reactions were carried out in 20 µL volumes consisting of: 1× FastStart SYBR Green Master; 0.2 µM each 86 bp, 0.4 µM each 190 bp, 0.45 µM each 452 bp, or 0.3 µM each IPC forward/reverse primer set; 2 µL sample or standard DNA; and PCR-grade water. For IPC assays, reactions included 0.5 µL diluted IPC (1×10^{-7} µM), for a final concentration of 2.5×10^{-9} µM.

Standard curves comprised an eight-point, 10× dilution series from 1×10^{10} to 10 mtDNA copies for each target. Each dilution was performed in duplicate (two intra-assay analyses) and evaluated over eight separate assays (eight inter-assay analyses), for a total of 16 analyses at each dilution.

Agarose gel electrophoresis

Amplification of each target from standard DNA and sample DNA (human buccal cell extract) was confirmed in duplicate by gel electrophoresis. PCR products were electrophoresed on a 1% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate (pH 8.5), 10 mM EDTA) with SYBR Safe DNA Gel Stain (Life Technologies, Carlsbad, USA). Product size was determined with reference to HyperLadder II (Bioline). Electrophoresis was carried out at approximately 6.3 V/cm with a PowerPac Basic (Bio-Rad, Hercules, USA) power supply. Gels were visualized in a Molecular Imager Gel Doc XR (Bio-Rad) under ultraviolet (UV; 302 nm) transillumination.

Results and discussion

Specificity of mtDNA targets

Sanger sequencing of a single human buccal sample at each of the 86, 190 and 452 bp mtDNA targets demonstrated complete human homology when compared to the rCRS aligned in Geneious R7. An absence of sequence homology with non-human DNA was verified by a BLAST analysis of each of the three human mtDNA targets (data not shown).

Isolation of mtDNA standard

Successful amplification of the 2119 bp mtDNA standard was verified by visualization of a single band of the expected size on an agarose gel (data not shown). Fluorescence measurements (n = 5) by Qubit dsDNA assay confirmed a highly concentrated (162 ± 5 ng/µL; mean ± 95% confidence interval) mtDNA standard. Purity of the mtDNA standard based on duplicate nucleic acid UV absorbance measures at 260 nm relative to absorbance at 280 nm (2.40 ± 0.01) and 230 nm (2.88 ± 0), revealed the likely absence of protein contaminants and inhibitors. Absorbance ratios beyond the generally accepted values for pure DNA (A260/280 ~ 1.8 and A260/230 ~ 1.8-2.2) is possible from RNA carry over or elution in alkaline buffer [18]. The presence of such carry over does not affect quantification approaches based upon dsDNA-specific Qubit reagent nor targeted qPCR.

Validation of the qPCR assays

Assay performance was evaluated in accordance with the MIQE guidelines on PCR efficiency (90–110%), linear dynamic range (coefficient of determination (R^2) ≥ 0.980), limit of detection (LOD; lowest dilution with ≥95% amplification success), accuracy (relative error of the mean copy number estimate) and precision (95% confidence interval) of calibration curves.

Reliable quantification was established with a linear range (R^2 ≥ 0.999) from 1×10^{10} to 10 copies per reaction, with repeatable and reproducible amplification success to the lowermost dilution in all instances (LOD of 10 copies) over duplicate intra-assay and eight inter-assay analyses. While a preliminary
duplicate analysis revealed detection was possible with satisfactory linearity (R^2 ≥ 0.987; data not shown) in a single copy, each dilution were excluded from subsequent analyses due to stochastic limitations of low-copy PCR [15], which introduced greater quantitative inaccuracies throughout the calibration series (up to 250% at single copy dilutions and 50% at non-single copy dilutions; data not shown). For the final calibration target, average amplification efficiencies were 98% for both the 86 and 190 bp amplicons and 88% for the 452 bp amplicon (Fig. 1). A reduction in PCR efficiency can be expected for longer targets due to the lower, combined annealing/extension temperature leading to suboptimal (slower) extension [19].

Assay accuracy was determined as the copy number estimate relative to the corresponding target copy number for each dilution, while precision was determined from the 95% confidence interval for each dilution as a fraction of the mean (Fig. 2). On average, each dilution of the 86 and 190 bp amplicons varied by less than 10% from the target copy number, with precision also within ±10% for all but the lowest dilution targeting 190 bp (±13%). Mean variances from target copy numbers near 10% were associated with the largest amplicon (452 bp) at most dilutions, which was increased at the uppermost (1×10^5 copies; 25%), middle (1×10^3 and 1×10^4 copies; 19%) and lowermost (10 copies; 18%) dilutions, with precision near ±10% at all but the lowest dilution (±15%). Reduced quantitative accuracy and precision of the longest amplicon, as compared to shorter targets, may accompany its less efficient PCR.

To confirm amplification success of sample DNA and IPC dilution series, single source human DNA was applied to the singleplex qPCRs and products visualized on an agarose gel (data not shown). Each mDNA target and IPC was successfully amplified in all cases within expected size ranges and without co-amplified product. Given that amplicon size can influence amplification efficiency and quantification cycle (C_q) in the presence of certain inhibitors [20], such inhibition controls are essential to the validity of these degradation assays.

Successful amplification by qPCR was achieved for IPC concentrations ranging 2.5×10^{-3} to 2.5×10^{-6}M; higher concentrated IPCs failed due to possible ‘over-fluorescence’ (data not shown). These lower concentrated IPCs can be applied as
Conclusion

Three human-specific mtDNA regions (86, 190 and 452 bp) were amplified by singleplex qPCR assays to provide an index of DNA degradation for modern, degraded or ancient samples. The developed assays were shown to be human specific, sensitive (LOD of 10 copies), accurate (within 10% relative error, for most target dilutions) and precise (within ±10% at 95% confidence, for most target dilutions). Quantification is possible from a standard curve of purified mtDNA standard with a linear dynamic range ($R^2 \geq 0.999$) of 1 × 10^4 copies down to 10 copies. The inclusion of an IPC assay allows detection of inhibitors for identification of samples requiring further clean-up, and to ensure the validity of degradative assays. Together, these assays provide invaluable information on both the quantity and quality of human mtDNA within a sample. While these assays would benefit from incorporation into a hydrolysis probe-based multiplex assay, singleplex SYBR Green assays are a highly cost effective alternative, provided enough sample is available for each reaction. These assays are recommended for mtDNA samples of limited quality in order to identify optimal downstream analyses and the likelihood of obtaining a successful result.

Key points

1. An index of mtDNA integrity can be determined from the quantification of two or more qPCR amplicons of increasing length.
2. Singleplex qPCR assays targeted three human-specific mtDNA amplicons located within the 12S rRNA and 16S rRNA sections of the mtDNA coding region, as well as a synthetic IPC oligonucleotide to monitor PCR inhibition.
3. Quantification was based on standard curves made from a purified mtDNA standard containing all three amplicons of interest.
4. The reported assays were human specific, sensitive (LOD of 10 mtDNA copies), accurate (within 10% relative error, for most target dilutions) and precise (within ±10% at 95% confidence, for most target dilutions), with a linear dynamic range ($R^2 \geq 0.999$) of 1 × 10^4 copies to 10 copies per reaction.

Acknowledgements

This research was supported by an Australian Institute of Nuclear Science and Engineering (AINSE) research award (ALNISTU18196) and an Australian Government Research Training Program (RTP) Scholarship.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approval for the collection and use of human biological material was granted by the University of Canberra Committee for Ethics in Human Research (Project Number 14-70). This article does not contain any studies with animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

References

2.3 Unpublished supplementary material

In addition to the published material (Section 2.2), an optimised procedure for the amplification of a mtDNA standard containing all three targets of interest, which was applied for the generation of standard curves as per the published manuscript, was developed. This was performed by assaying products amplified with MyTaq DNA Polymerase (Bioline, London, UK) under various thermal cycling conditions by agarose gel electrophoresis. Amplification success for each of the mtDNA targets from the purified standard and from human DNA extract was also verified, as was amplification of the IPC. In summary, the following strategy was employed to develop the assay:

1. Optimise the amplification of a human mtDNA standard incorporating all three assay targets, including use of:
   a. Two different primer sets (for a 1910 or 2119 bp mtDNA standard)
   b. Three different thermal cycling protocols (the standard MyTaq DNA Polymerase protocol, the original ACAD multiplex protocol, or an amalgamation of both protocols)

2. Optimise an assay suitable for mtDNA quantity and quality determination, including:
   a. Purification of the human mtDNA standard after application of the optimised amplification approach
   b. Verification of amplification success for the three mtDNA targets using the purified mtDNA standard and human DNA extract, including preliminary generation of real-time PCR standard curve amplification plots
   c. Determination of an appropriate IPC concentration to accompany the mtDNA degradation assays

2.3.1 Methodology

2.3.1.1 General laboratory and chemical preparation

To limit potential sources of contamination, work areas were treated with 10 % sodium hypochlorite (DNA-free areas only) and/or 70 % ethanol with UV-irradiation for 30 minutes before and after each use. Plasticware and buffers were sterilised by autoclaving in a 3870EL Autoclave (Tuttnauer, Breda, Netherlands) at 120 kilopascals (kPa) and 121 ºC for 15 minutes
(wrapped instruments) or 20 minutes (liquids), and by 30 min UV-irradiation, prior to use. A separate DNA-free hood was used for PCR setup, with non-amplified DNA being handled only within a laminar flow cabinet. A negatively pressurised post-amplification laboratory was used to isolate amplified product from non-amplified DNA. Disposable gloves and clean laboratory coats were worn at all times.

TE buffer (10 millimolar (mM) Tris-chloride, pH 8.0, 0.1 mM EDTA) was prepared with Trizma base (Sigma-Aldrich, St. Louis, USA) and EDTA disodium salt dihydrate (Mallinckrodt, Surrey, UK) with hydrochloric acid (32 % w/w; Chem-Supply, Gillman, Australia) for pH adjustment.

### 2.3.1.2 DNA samples and targets

DNA was extracted from human buccal cells collected from a single donor using the ChargeSwitch Forensic DNA Purification Kit (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol (Invitrogen 2005). Purified DNA was quantified using the Qubit dsDNA HS Assay Kit (Invitrogen) with a Qubit 2.0 Fluorometer (Invitrogen). Targets included a 2119 bp mtDNA standard generated from primer sequences flanking the three mtDNA degradation targets, a 1910 bp mtDNA standard generated using the two furthest mtDNA degradation target primer sequences (190 bp forward and 86 bp reverse primer), as well as the 86, 190 and 452 bp mtDNA degradation targets and an IPC oligonucleotide. Primer and other oligonucleotide sequences are as detailed in Section 2.2.2.

### 2.3.1.3 Optimisation of an amplification procedure for the isolation of a mitochondrial DNA standard

Amplification of the mtDNA standard was performed using MyTaq DNA Polymerase (Bioline). PCR component concentrations were applied as published (Section 2.2.2). The 190 bp target was also amplified as a control to indicate amplification success of shorter amplicons. A series of PCR thermal cycling conditions were assayed (Table 2.1) based on the manufacturer protocol (Bioline n.d.) for MyTaq (Method A), the ACAD multiplex protocol applied for real-time PCR of mtDNA degradation targets (Method B), and an amalgamation of both methods (Method C). PCR products were visualised on a 1 % (w/v) agarose gel in accordance with the published methodology (Section 2.2.2).
Table 2.1: Thermal profiles applied to optimisation experiments for amplification of a mitochondrial DNA standard using a GeneAmp PCR System 9700.

<table>
<thead>
<tr>
<th>Reaction step</th>
<th>Method A</th>
<th>Method B</th>
<th>Method C</th>
</tr>
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<tr>
<td><strong>Stage 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activation/denaturation</td>
<td>95 ºC, 1 minute</td>
<td>95 ºC, 10 minutes</td>
<td>95 ºC, 10 minutes</td>
</tr>
<tr>
<td><strong>Stage 2 (35 cycles)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 ºC, 15 seconds</td>
<td>95 ºC, 15 seconds</td>
<td>95 ºC, 15 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>60 ºC, 15 seconds</td>
<td>60 ºC, 100 seconds</td>
<td>60 ºC, 30 seconds</td>
</tr>
<tr>
<td>Extension(^a)</td>
<td>72 ºC, 1 minute</td>
<td></td>
<td>72 ºC, 1 minute</td>
</tr>
<tr>
<td><strong>Stage 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final extension(^b)</td>
<td>72 ºC, 10 minutes</td>
<td>60 ºC, 10 minutes</td>
<td>72 ºC, 10 minutes</td>
</tr>
</tbody>
</table>

\(^a\) Cycling extension time at 72 ºC was 30 seconds for the 190 base pair control target

\(^b\) Final extension time was 1 minute for the 190 base pair control target

2.3.1.4 Standard purification

The final mtDNA standard was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), as previously described (Section 2.2.2). The quality of the purified mtDNA standard was checked via agarose gel electrophoresis and spectrophotometry, and quantity determined fluorometrically, as previously described (Section 2.2.2).

2.3.1.5 Real-time PCR and internal PCR control validation

Real-time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems) using the published methodology (Section 2.2.2), with the exception that the PCR was cycled 50 times. Each of the three mtDNA degradation targets were amplified from human DNA extract and mtDNA standard. To determine a suitable IPC concentration for application to the mtDNA degradation assays, a 10× dilution series of the IPC oligonucleotide was amplified by real-time PCR, with final concentrations ranging from 2.5×10⁻² to 2.5×10⁻⁹ µM. Amplification of each target from a human buccal extract or synthetic IPC oligonucleotide was visualised by agarose gel electrophoresis, as described previously (Section 2.2.2). A preliminary 10× dilution series of the mtDNA standard, from 1×10⁷ copies to a single copy of each target, as well as the IPC dilution series, were additionally examined by real-time amplification plots.
2.3.2 Results

2.3.2.1 Optimisation of an amplification procedure for a mitochondrial DNA standard

The MyTaq DNA Polymerase chemistry was unsuccessfully applied for the amplification of all targets when using the standard thermal cycling protocol (Method A), as visualised on an agarose gel (Figure 2.1). However, successful amplification of shorter targets was attained when using the ACAD multiplex thermal cycling conditions (Method B) and an amalgamated thermal profile (Method C). The 2119 bp mtDNA standard produced a visible product at expected size range only when the amalgamated method was applied. The 1910 bp mtDNA standard, using the two furthest mtDNA degradation target primers, could not be successfully amplified under any of the tested conditions.

Figure 2.1: Optimisation of the mitochondrial DNA standard amplification procedure with MyTaq DNA Polymerase. DNA was amplified in a GeneAmp PCR System 9700 and visualised on a 1 % (w/v) agarose gel with SYBR Safe DNA Gel Stain under ultraviolet transillumination. Each panel includes DNA ladder (HyperLadder II, lanes 1 and 15). Panel A additionally includes the 190 bp target (lanes 5 and 6), 1910 bp standard (lanes 8 and 9), and 2119 bp standard (lanes 11 and 12) amplified with the ‘Method A’ thermal profile; and negative controls (lanes 4, 7, 10 and 13). Panel B includes the 190 bp target amplified with the ‘Method B’ thermal profile (lanes 2 and 3) or ‘Method C’ thermal profile (lanes 7 and 8); and negative control (lane 5). Panel C includes the 1910 bp standard (lanes 2 and 3) and 2119 bp standard (lanes 8 and 9) with the ‘Method B’ thermal profile; the 1910 bp standard (lanes 10 and 11) and 2119 bp standard (lanes 13 and 14) with the ‘Method C’ thermal profile; and negative control (lane 5).
2.3.2.2 Standard purification

The mtDNA standard was amplified and purified, as well as quantified and qualified, according to the published methods (Section 2.2.2). The 2119 bp standard was visualised by agarose gel electrophoresis, which revealed a bright band at the expected size (Figure 2.2).

![Gel electrophoresis of the purified mitochondrial DNA standard.](image)

**Figure 2.2:** Gel electrophoresis of the purified mitochondrial DNA standard. The standard was purified from single source human DNA using MyTaq DNA Polymerase in a GeneAmp PCR System 9700 and the QIAquick PCR Purification Kit. Products were visualised on a 1 % (w/v) agarose gel with SYBR Safe DNA Gel Stain under ultraviolet transillumination. DNA ladder (HyperLadder II, lane 1), negative control (lane 3), and the purified 2119 bp mtDNA standard (lane 5) were assayed.

2.3.2.3 Real-time PCR and internal PCR control validation

The mtDNA degradation targets were amplified from a human buccal extract by real-time PCR, as was the IPC dilution series, as described previously (Section 2.2.2). Successful amplification of each mtDNA target, in duplicate, and diluted IPC, was attained in all cases with products electrophoresed as single bands at expected size (Figure 2.3). Upon examination of real-time amplification plots (Figure 2.4), a preliminary amplification of mtDNA standard curves for each target, diluted 10× from 1×10^7 copies, was possible down to a single copy, with even spacing between the exponential phase of the dilution series and precise replicate plots down to 10 copies. Amplification of the IPCs concentrated at and above 2.5×10^{-4} μM in the final reaction could not be detected by real-time PCR plots. All amplified targets plateaued before 40 cycles, and detectable amplification of negative control reactions occurred after this point.
Figure 2.3: Gel electrophoresis of singleplex real-time PCR targets. Panel A includes DNA ladder (HyperLadder II, lane 1), duplicate mitochondrial DNA targets (86 base pairs (bp), lanes 2 and 3; 190 bp, lanes 4 and 5; 452 bp, lanes 6 and 7), high concentration internal PCR control (IPC) dilutions (1 µM, lane 8; 0.1 µM, lane 9) and negative control (lane 10). Panel B includes DNA ladder (lane 1), remaining 10× serially diluted IPCs (0.01 to 1×10⁻⁷ µM, lanes 2 to 7) and negative control (lane 8). Products were generated from single source human DNA or synthetic IPC oligonucleotide using FastStart SYBR Green Master and a 7500 Real-Time PCR System, visualised on a 1 % (w/v) agarose gel with SYBR Safe DNA Gel Stain under ultraviolet transillumination.

Figure 2.4: Real-time PCR amplification plots of mitochondrial DNA (mtDNA) targets. Amplification was by a 7500 Real-Time PCR System with SYBR Green. A 10× dilution series from 1×10⁷ to a single copy of the mtDNA standard was assayed in duplicate for the (A) 86 base pair (bp) amplicon, (B) 190 bp amplicon, and (C) 452 bp amplicon. The internal PCR control (IPC) was 10× serially diluted from 2.5×10⁻⁵ to 2.5×10⁻⁹ µM (D). A negative control was also assayed in duplicate (E).
2.3.3 Discussion

Amplification of a mtDNA standard was attempted under various conditions, including the use of different primer sets and thermal cycling protocols using MyTaq DNA Polymerase (Section 2.3.2.1). This kit is suitable for the amplification of targets up to 5 kilobases (kb) in length. Optimal concentrations of DNA polymerase are recommended by the kit manufacturer and PCR components (dNTPs, Mg\(^{2+}\) and enhancers) are supplied in a premixed master buffer at pre-optimised concentrations; thus, these components did not require further optimisation. The MyTaq reaction chemistry successfully amplified the 2119 bp mtDNA standard when applied to a modified thermal cycling procedure, which included facets of both the standard MyTaq and ACAD multiplex protocols (Figure 2.1). This same procedure could not amplify the 1910 bp standard from the two furthest mtDNA degradation target primers. However, amplification of a longer standard using dedicated primers is a superior method, since all qPCR target primer binding sites are located away from the amplicon ends. This will accommodate for a reduced polymerase efficiency toward the amplicon ends that could be expected during extension of a long template (Barnes 1994). This will minimise any loss of PCR efficiency within primer binding sites for qPCR targets.

The 2119 bp amplicon produced a single, extremely bright band at the anticipated size range, which was suggestive of a highly concentrated and pure mtDNA standard (Figure 2.2), as verified by spectrophotometry and fluorometry (Section 2.2.2). This purified standard was applied successfully for amplification of mtDNA degradation targets, which were also able to be amplified from a human DNA extract (Figure 2.3). Amplification of mtDNA targets from the mtDNA standard was possible down to a single copy; however, stochastic variation resulted in uneven spacing of the exponential phase with respect to more concentrated dilutions (reduced linearity), as well as reduced duplicate precision (Figure 2.4A, B and C). Thus, dilution down to 10 copies was selected for the standard curves (Section 2.2.2). While product amplified from the IPC dilution series by real-time PCR was detectable at all dilutions on an agarose gel (Figure 2.3), only dilutions at and below 2.5×10^{-5} \mu M produced adequate amplification curves (Figure 2.4D and E). Therefore, while the PCR was successful at higher concentrations, dilution of the IPC is necessary to prevent detection problems (e.g. due to over-fluorescence) by real-time instruments. Thermal cycling beyond 40 cycles was considered unnecessary due to the PCR reaching its plateau phase for each target by this point (Figure 2.4). This was further justified by detection of amplified product in one of the negative controls after 40 cycles. Fewer cycles would not have allowed for complete amplification of the lowest standard dilution.
2.3.4 Summary and conclusions

This work verified the potential to apply SYBR Green for the singleplex amplification and quantification of three mtDNA targets of different length. A mtDNA amplicon containing all three targets could be successfully purified for use as a mtDNA standard, which was detectable down to a single copy. However, for use as a qPCR standard, dilution down to 10 copies appeared to be more stable for quantification purpose. A synthetic IPC was amplified and detected by real-time PCR with success from $2.5 \times 10^{-5}$ to $2.5 \times 10^{-9}$ µM. The developmental and validation experiments described herein laid the foundation for the published manuscript (Section 2.2.2). The final assay was suitable for the evaluation of mtDNA degradation after gamma-irradiation (Chapter 3).
Chapter 3: Effects of gamma-irradiation on nuclear versus mitochondrial DNA and forensic genotyping
3.1 Introduction

High-dose exposure of DNA evidence to gamma-radiation may be caused by gamma-emitting radionuclides present at a radiological crime. Such crimes may involve the abandonment, theft, or trafficking of radioactive material that can be used for the construction of crude radiological weapons, such as a RED or RDD (Ferguson et al. 2003). The placement of DNA evidence within proximity of a radioactive source for any length of time may result in its exposure to substantial doses of ionising radiation. Gamma-irradiation may also be applied to decontaminate biological agents from forensic samples (Hoile et al. 2010). Due to its high probative value, DNA evidence is the most reliable means of identification available today, and hence may be critical for the identification of victims or perpetrators of such crimes.

Genotyping of autosomal STRs, located within nuDNA, is the current standard for forensic identity testing (Section 1.2.2.1), together with part of the X-Y homologous amelogenin gene for sex-indication (Jeffreys 2005; Sullivan et al. 1993). STRs are sites of length polymorphisms, which vary with high frequency between individuals (Kimpton et al. 1994; Kimpton et al. 1993). Genotyping involves the in vitro amplification of targets via PCR, which requires the targeted loci to be undamaged, or intact (Section 1.2.6.2). Ionising irradiation of DNA evidence can introduce a variety of DNA lesions analogous to degraded DNA, including base modifications (e.g. oxidation, deamination), abasic sites, crosslinkages, and strand breaks (Section 1.3.2.1). These lesions impede the PCR through prevention of strand uncoiling, misrecognition of primer binding sites, and/or blockage of DNA polymerase (Matuo et al. 2013; Sikorsky et al. 2007).

The STR genotype can become compromised (i.e. leading to allelic dropout) following sufficiently high doses of gamma-radiation. A negative correlation has previously been reported between dose and STR genotyping success, especially beyond doses of 10 kGy (Hodgson & Baxter 2013; Hoile et al. 2010). Resultant allele dropout from DNA degradation (Section 1.2.6.2) is particularly noticeable for longer amplicons that incur DNA damage lesions with greater frequency (Takahashi et al. 1997). Gamma-radiation interacts with DNA constituents via direct ionisation events, as well as through secondary oxidative reactions mediated by ROS (Section 1.3.1). The latter are produced from the radiolysis of intra/extracellular water molecules and mitochondrial hyperfunction (Das 2013; Dizdaroglu et al. 2002; Hutchinson 1985; Leach et al. 2001; Yamamori et al. 2012).
Due to its high copy number per cell, mtDNA is more likely to be recovered intact and contains sequence polymorphic HVRs (Section 1.2.2.2) within the control region (D-loop). Sequencing of mitochondrial HVRs is an alternative to STR genotyping for degraded DNA and, while the discrimination power of multiplexed STRs for identification of an individual is unrivalled by HVR sequencing, enables identification from maternal lineage (Budowle et al. 2000; Mabuchi et al. 2007). However, given the role of the mitochondria in mediating ROS production, mtDNA is subjected to greater oxidative damage than nuDNA (Morales et al. 1998; Richter et al. 1988; Yakes & van Houten 1997). Mitochondrial content and oxidative function may also be upregulated by ionising radiation exposure (Leach et al. 2001; Yamamori et al. 2012), and the mitochondria may be subjected to more frequent ionisation events than the nucleus, depending on mitochondrial volume (Kam et al. 2013).

The aim of this study was to evaluate the degradation of STR genotypes after high doses (1-50 kGy) of gamma-irradiation to both liquid (hydrated) and dried (dehydrated) whole blood samples, as well as the relative impact of gamma-irradiation upon nuDNA and mtDNA targets. Integrity indices for mtDNA were determined from the quantity ratios of different sized amplicons targeted by qPCR assays (Section 2.2.2). Similarly, peak height ratios between STRs of equivalent size to the mtDNA targets were used to provide an index of nuDNA integrity.

### 3.2 Methodology

#### 3.2.1 General laboratory and chemical preparation

Laboratory areas, equipment and TE buffer were prepared in accordance with Section 2.3.1.1.

#### 3.2.2 DNA samples

Whole blood was collected by venepuncture from 10 individuals into 4 mL Vacutainers (Becton Dickinson, Franklin Lakes, USA) coated with 7.2 milligrams (mg) of dipotassium EDTA. Aliquots of 150 µL were transferred into 1.5 mL glass vials with polyethylene push caps (liquid samples) or air dried onto glass microscope slides (dried samples). Slides were previously sterilised with 70 % ethanol and a sterile glass cover slip was secured over dried blood flakes with adhesive tape. Blood collection and sample preparation was performed for non-irradiated
controls and irradiated samples the day prior to sample irradiation and stored at 4 °C until irradiation.

### 3.2.3 Sample irradiation

Gamma-irradiation of whole blood samples was conducted at the Australian Nuclear Science and Technology Organisation (ANSTO) using the Gamma Technology Research Irradiator (GATRI). Irradiations with $^{60}$Co to approximate absorbed doses of 1, 5, 10, 25 and 50 kGy were performed independently at ambient temperature (~24.0 °C). For each dose, sample slides and vials were placed in a 30 millimetre (mm) thick polystyrene box and the dose rate confirmed from the presence of two ceric-cerous sulphate dosimeters. An exception was the 1 kGy samples, which relied on a dose rate previously determined by a dose mapping study (data not shown). Samples were positioned within the GATRI chamber to deliver a dose rate of approximately 2 kGy per hour over a continuous period until the target dose was reached. Therefore, exposure times varied for each dose from approximately 30 minutes (1 kGy) to 24 hours (50 kGy). A correction for the decay of the radioactive source was employed in calculating exposure times. Samples were immediately stored at -20 °C post-irradiation.

Sample irradiations took place over a period of three days in separate batches for each dose. To evaluate any impact of storage time on DNA integrity, two sets of unirradiated controls were prepared for each individual and sample type (i.e. liquid or dried). These controls were stored under the same conditions as either the first or last irradiation batch for subsequent comparison of DNA integrity.

### 3.2.4 DNA extraction

Whole blood samples were extracted using the QIAamp DNA Mini Kit (Qiagen) spin protocol for blood and body fluids, following the manufacturer’s guidelines (Qiagen 2016). Sample lysis was carried out directly in the glass vials of liquid samples or by scraping the dried blood flakes with a sterile scalpel into 1.5 mL microcentrifuge tubes. Extracted DNA was eluted into 100 µL elution buffer (10 mM Tris-chloride, pH 9.0, 0.5 mM EDTA). Aliquots of the DNA extracts were stored at -20 °C prior to use.
3.2.5 Quantitative real-time PCR (qPCR)

Quantification of a 62 bp nuDNA target was performed with the Quantifiler Human DNA Quantification Kit (Applied Biosystems) following the manufacturer’s protocol (Applied Biosystems 2018). Three mtDNA targets of different length (86, 190 and 452 bp) were quantified by SYBR Green-based qPCR assays, according to Section 2.2.2. All assays were performed on a 7500 Real-Time PCR System with HID Real-Time PCR Analysis Software v1.1 (Applied Biosystems). IPCs were included with both the Quantifiler and mtDNA assays to verify the presence or absence of PCR inhibitors.

3.2.6 STR genotyping

A panel of 23 forensic STR markers and amelogenin were genotyped using the GlobalFiler PCR Amplification Kit (Applied Biosystems). The standard 25 µL reaction chemistry was applied (Applied Biosystems 2016), with products amplified from 1 ng template DNA (29 cycle protocol) on a Veriti Thermal Cycler (Applied Biosystems). Sample dilutions were in TE buffer (10 mM Tris-chloride, pH 8.0, 0.1 mM EDTA). Negative and positive controls were TE buffer and Control DNA 007 (Applied Biosystems), respectively, in place of sample DNA.

CE was performed according to the GlobalFiler protocol for plate setup (Applied Biosystems 2016), using GeneScan 600 LIZ dye Size Standard v2.0 (Applied Biosystems) and Hi-Di Formamide (Applied Biosystems). Electrophoresis was performed on a 3500xl Genetic Analyser with 3500 Series Data Collection Software 2 (Applied Biosystems). The capillary length was 36 cm filled with POP-4 Polymer (Applied Biosystems) and the standard run module was applied (HID36_POP4xl); oven temperature was 60 ºC, prerun conditions were 15 kilovolts (kV) for 180 seconds (s), injection conditions were 1.2 kV for 24 s, and run conditions were 13 kV for 1550 s, with a 1 s data delay. Spectral calibration was performed with DS-36 Matrix Standard (Dye Set J6; Applied Biosystems). Analysis of genotypes was conducted in GeneMapper ID-X v1.4 (Applied Biosystems) with a detection limit of 225 RFU. Stochastic thresholds for heterozygote and homozygote alleles were 500 and 1000 RFU, respectively. A heterozygote peak imbalance threshold of 70 % was employed across all loci.
3.2.7 DNA degradation assays

An index of DNA integrity was determined for both nuDNA and mtDNA from the amplification of long versus short targets. For nuDNA, a subset of autosomal forensic STR markers were selected for relative size consistency with the three mtDNA targets (86, 190 and 452 bp; Section 3.2.5), including loci of low molecular weight (D2S441, ~75-110 bp), intermediate molecular weight (vWA and D1S1656, ~150-210 bp), and high molecular weight (TPOX and SE33, ~310-450 bp). For STR size groups containing multiple loci, the average peak heights of alleles for each marker were calculated. Integrity indices were then calculated from the peak height or quantity ratios of increasing amplicon size difference, comprising intermediate/short (Index A), long/intermediate (Index B), and long/short loci (Index C).

3.2.8 Statistical analysis

To account for any variation between the two sets of unirradiated controls (stored under the same conditions as the first or last irradiation batch, described in Section 3.2.3), the irradiated samples were compared against both sets of controls and the data pooled for statistical analysis using SPSS Statistics 23 (IBM, Armonk, USA). A 5 % significance level was applied to all tests and asymptotic (two-tailed) p-values obtained, where a p-value of less than 0.05 was considered a statistically significant result. Divergence from a normal Gaussian distribution was checked using Shapiro-Wilk normality tests and confirmed by quantile-quantile (Q-Q) plots. Equality of variances were checked using Levene’s test. Extreme values were identified and removed from the data analysis if they were beyond the first or third quartile of the dataset by more than 1.5× the interquartile range.

Nonparametric tests for related samples (repeated measures) were applied for evaluation of any dose-effect differences within data grouped by integrity index (A, B or C) and sample preparation (hydrated or dehydrated), and between nuDNA and mtDNA. These analyses were conducted using Friedman’s tests, with multiple comparisons made by Wilcoxon Signed-Rank tests. Mann-Whitney U tests were used to compare the effect of sample hydration status at equivalent doses. Sequential Bonferroni (Holm-Bonferroni) corrected significance levels were employed to adjust p-values in order to mitigate against chance significance due to multiple comparisons.
3.3 Results

3.3.1 DNA quantification and PCR inhibitor detection

The presence of inhibitors was tested using a TaqMan-based IPC multiplexed with the Quantifiler chemistry applied to nuDNA and a separate SYBR Green-based IPC designed for use with the mtDNA assays. Both inhibitor assays indicated the absence of detectable inhibitors in all samples, with amplification of the IPC achieved after about 25 cycles using Quantifiler and 30 cycles using the SYBR Green IPC assay. This was consistent for samples of unknown quantity/inhibition, as well as uninhibited positive and negative controls (data not shown). The nuDNA concentrations of pooled unirradiated controls ranged from 10 to 34 ng/µL for dehydrated samples and 31 to 94 ng/µL for hydrated samples, while mtDNA ranged from 5000 to 90000 copies/µL (10 to 210 femtograms (fg)/µL) for dehydrated samples and 32000 to 150000 copies/µL (74 to 350 fg/µL) for hydrated samples (data not shown).

3.3.2 Integrity of unirradiated genotypes

Two sets of unirradiated controls were prepared for each of the 10 individuals and sample types (liquid or dried) for storage under the same conditions as the first or last irradiation batch, as described in Section 3.2.3. The duplicate unirradiated controls were pooled to compare the integrity of genotypes generated from hydrated and dehydrated samples prior to irradiation, with reference to four positive controls (Control DNA 007) used for STR genotyping (Figure 3.1). Mean peak heights (± 95 % confidence interval) of all controls were consistent, if not slightly higher for sample matrix controls, at 9200 ± 500 RFU (dried sample controls), 8900 ± 480 RFU (liquid sample controls) and 7300 ± 660 RFU (Control DNA 007). The variation of the two pooled sets of unirradiated controls was used to construct 95 % confidence intervals for each locus ranging from ± 10-28 % (dried sample controls) and ± 11-37 % (liquid sample controls) relative to the mean, which was consistent with the variation of the Control DNA 007 peak heights across loci (± 3-41 % relative confidence interval). However, while all heterozygous alleles were well balanced for Control DNA 007, heterozygote imbalance (< 70 % peak height ratio) was observed in half of the dried control samples (at up to two loci) and in 80 % of liquid controls (at up to three loci). No correlation with amplicon size was discernible; respective frequencies of imbalanced loci for hydrated and dehydrated samples
were 48 and 45 % below 200 bp, 10 and 27 % from 200 to 300 bp, and 43 and 36 % above 300 bp (data not shown).

**Figure 3.1: Peak heights and heterozygote balance of short tandem repeat (STR) genotypes for unirradiated samples and positive controls.** Dried (dehydrated) and liquid (hydrated) whole blood samples prepared in duplicate for 10 individuals (n = 20 for autosomal STRs; n = 10 for Y STRs) were genotyped using the GlobalFiler PCR Amplification Kit. Positive controls (n = 4) consisted of Control DNA 007. At each locus, allelic peak heights, measured in relative fluorescence units (RFU), were averaged across samples and the mean ± 95 % confidence interval reported (bars: left vertical axis). Heterozygote balance was calculated as a percentage ratio of the smaller to taller peak and the median ± minimum/maximum reported (dots: right vertical axis) against an imbalance threshold of 70 % (indicated by the solid line). STR loci are arranged in approximate size order (Y indel < SE33).
3.3.3 Effect of gamma-irradiation on forensic STR genotyping

A panel of STRs were amplified from whole blood (hydrated and dehydrated) using the GlobalFiler PCR Amplification Kit and evaluated for signs of degradation after high-dose (1-50 kGy) gamma-irradiation (Figure 3.2). Peak height averages (± 95 % confidence interval) across all loci for 10 individuals did not differ substantially for dehydrated samples at 1 kGy (11000 ± 750 RFU), 5 kGy (12000 ± 760 RFU), or 10 kGy (12000 ± 790 RFU). A slight decline was evident at 25 kGy (9700 ± 680 RFU), which preceded a noticeable peak height reduction, particularly for longer amplicons, at 50 kGy (5800 ± 470 RFU). Similarly, the averaged peak height of hydrated samples was relatively consistent between 1 kGy (13000 ± 820 RFU) and 5 kGy (11000 ± 690 RFU), declining slightly at 10 kGy (9100 ± 622 RFU), and further deteriorating at 25 kGy (5800 ± 530 RFU) and 50 kGy (3000 ± 400 RFU).
Figure 3.2: Peak heights and heterozygote balance of short tandem repeat (STR) genotypes for irradiated samples. Gamma-irradiation of dried (dehydrated: left) and liquid (hydrated: right) whole blood was performed at doses of 1, 5, 10, 25 and 50 kilogram (kGy). Samples from up to 10 individuals (9 ≤ n ≤ 10 for autosomal STRs; n = 5 for Y STRs) were genotyped using the GlobalFiler PCR Amplification Kit. At each locus, allelic peak heights, measured in relative fluorescence units (RFU), were averaged across samples and the mean ± 95% confidence interval reported (bars: left vertical axis). Heterozygote imbalance was calculated as a percentage ratio of the smaller to taller peak and the median ± minimum/maximum reported (dots: right vertical axis) against an imbalance threshold of 70% (indicated by the solid line). STR loci are arranged in approximate size order (Y indel < SE33).
Significant changes in the peak heights of loci after irradiation were apparent relative to the unirradiated controls (Figure 3.3). TH01, D2S1338 and DYS391 produced markedly higher relative changes in comparison to other loci, consistent with unirradiated controls exhibiting lower peak heights at these loci; thus, these loci were excluded from statistical analysis. A significant increase in the averaged (± 95 % confidence interval) locus peak height was observed at 1 kGy for hydrated (by 41 ± 3.99 %, p < 0.000) and dehydrated samples (by 18 ± 2.93 %, p < 0.000), 5 kGy for hydrated (by 24 ± 3.92 %, p < 0.000) and dehydrated samples (by 34 ± 3.71 %, p < 0.000), and 10 kGy for dehydrated samples only (by 31 ± 3.70 %, p < 0.000). Overall peak heights fell to within the variation observed in pooled controls at 10 kGy for hydrated samples (p = 0.260) and 25 kGy for dehydrated samples (p = 0.299). A significant decline in peak height below that of the controls occurred for hydrated samples at 25 kGy (by 34.6 ± 2.79 %, p < 0.000) and 50 kGy (by 67.5 ± 2.53 %, p < 0.000), which did not occur for dehydrated samples until 50 kGy (by 37.3 ± 2.29 %, p < 0.000). Likewise, the frequency of peak height increases for dehydrated samples were reasonably consistent from 1 to 10 kGy (around 70 to 85 %), but steadily declined as the dose increased, down to 42 and 8 % of cases at 25 and 50 kGy, respectively. For hydrated samples, a larger proportion of loci increased in peak height at 1 kGy (92 %), with the frequency of peak height increases becoming more rapidly and steadily reduced beyond this dose (around 70 % at 5 kGy, 40 % at 10 kGy, and 10 % at 25 kGy), down to only 3 % of cases at 50 kGy.
Figure 3.3: (A) Change in the peak height of short tandem repeat (STR) loci for irradiated samples relative to unirradiated controls, and (B) the frequency of loci that increased in relative peak heights. Gamma-irradiation of dried (dehydrated) and liquid (hydrated) whole blood was performed to doses of 1, 5, 10, 25 and 50 kilogray (kGy). Samples from up to 10 individuals were genotyped using the GlobalFiler PCR Amplification Kit for comparison with each of two duplicate sets of unirradiated controls, prior to pooling of both data sets (16 ≤ n ≤ 20 for autosomal STRs; 9 ≤ n ≤ 10 for Y STRs). At each locus, allelic peak heights, measured in relative fluorescence units (RFU), were averaged for determination of relative change from the unirradiated controls (indicated by the solid line at 0 %) and the mean ± 95 % confidence interval reported (Figure A), along with the overall frequency of loci that increased in relative peak height (Figure B). STR loci are arranged in approximate size order (Y indel < SE33).
Employing stochastic heterozygote and homozygote thresholds of 500 and 1000 RFU, respectively, full profiles were attained for all dehydrated samples, as well as hydrated samples up to 25 kGy. Partial profiles, consisting of peaks below peak height thresholds, were only attained for 50 kGy hydrated samples, although the mean (± 95 % confidence interval) frequency of above-threshold alleles remained at 86.4 ± 4.10 % at this dose. Allelic peak heights below peak height thresholds (dropout) occurred only at loci with size ranges above approximately 225 bp (D16S539 to SE33), with completely undetectable alleles (below 225 RFU) in 5.3 % of these loci (all at SE33). Genotype non-concordance was found at nonreportable (subthreshold) loci with a frequency of 21 %, where dropout occurred at a single heterozygous locus, leading to the false calling of a homozygote.

Heterozygote imbalance (Figure 3.2) contributed to greater levels of nonreportable alleles, particularly as dose increased. No cases of imbalance were observed in dehydrated samples at 1 kGy, with 1-3 cases (10-30 % of samples) demonstrating imbalance at up to two loci each from 5 to 25 kGy and seven cases (70 % of samples) with imbalance at up to four loci each at 50 kGy. Hydrated samples exhibited higher rates of imbalance, with observations of 3-4 cases (30-40 % of samples) with imbalance at up to two loci each from 1 to 10 kGy, nine cases (90 % of samples) with up to three imbalanced loci at 25 kGy, and eight cases (80 % of samples) with 2-6 imbalanced loci at 50 kGy. In comparison to the unirradiated controls, fewer imbalances were found after exposure to lower doses, with a 40-100 % reduction in imbalanced dehydrated samples between 1-25 kGy and 50-63 % reduction in imbalanced hydrated samples between 1-10 kGy. Only samples irradiated at doses above these ranges had higher frequencies of imbalances than the unirradiated controls, with imbalances occurring in 40 % more dehydrated samples at 50 kGy, but only in up to 13 % more hydrated samples at 25 to 50 kGy. Imbalances predominantly affected amplicons above 200-300 bp.

3.3.4 Effect of gamma-irradiation on nuDNA integrity

The nuDNA integrity indices (Section 3.2.7) after irradiation were expressed as a percentage relative to the unirradiated controls (‘relative integrity index’; Figure 3.4). Most samples demonstrated a relative increase in their integrity index at lower doses (≤ 5 kGy), which was not the case as dose increased. Over all integrity indices, dehydrated samples were increased relative to unirradiated controls in more cases than not at 1 kGy (77 % of cases) and 5 kGy (56 % of cases), with increases in 43 % of cases at 10 kGy, 22 % of cases at 25 kGy, and only 2 %
of cases at 50 kGy. Hydrated samples exhibited relative increases in integrity index in similar proportion to dehydrated samples at 1 kGy (78 % of cases), with increases in 42 % of cases at 5 kGy, 21 % of cases at 10 kGy, and no cases after 25 kGy.

Figure 3.4: Nuclear DNA integrity indices as a percentage relative to those for unirradiated controls at each dose. Gamma-irradiation of dried (dehydrated: left) and liquid (hydrated: right) whole blood was performed to doses of 1, 5, 10, 25 and 50 kilogray (kGy). Samples from up to 10 individuals were genotyped using the GlobalFiler PCR Amplification Kit for comparison with each of two duplicate sets of unirradiated controls, prior to pooling of both data sets (18 ≤ n ≤ 20). Integrity indices comprised of averaged peak height ratios for intermediate/short (Index A), long/intermediate (Index B), and long/short loci (Index C), expressed as percentages relative to those for unirradiated controls (indicated by the solid line at 100 %). Extreme values (points) are 1.5× the interquartile range beyond the first or third quartile and are labelled with the corresponding donor number.

Most of the averaged (± 95 % confidence interval) integrity indices were significantly increased relative to unirradiated controls for dehydrated samples at 1 kGy (Index B, by 9.61 ± 3.98 %, p = 0.005; Index C, by 17.0 ± 7.73 %, p = 0.005) and 5 kGy (Index B, by 14.1 ± 4.27 %, p = 0.003), with no significant changes at 10 kGy (Figure 3.5). For hydrated samples, significant increases in integrity index from controls were only observed at 1 kGy (Index B, by 7.84 ± 5.40 %, p = 0.019; Index C, by 17.2 ± 8.03 %, p = 0.001), with no significant changes at 5 kGy. As dose increased, more substantial declines in relative integrity index occurred, up to 17.7 ± 4.63 % (Index A, p = 0.003), 25.0 ± 4.44 % (Index B, p = 0.001), and 37.5 ± 4.74 % (Index C, p = 0.001) for dehydrated samples at 50 kGy, and up to 58.1 ± 2.46 % (Index A, p = 0.002), 76.3 ±
3.59 % (Index B, \( p = 0.001 \)), and 89.1 ± 1.94 % (Index C, \( p = 0.001 \)) for hydrated samples at 50 kGy.

Figure 3.5: Change in nuclear DNA integrity indices relative to those for unirradiated controls at each dose. Gamma-irradiation of dried (dehydrated: left) and liquid (hydrated: right) whole blood was performed to doses of 1, 5, 10, 25 and 50 kilogram (kGy). Samples from up to 10 individuals were genotyped using the GlobalFiler PCR Amplification Kit for comparison with each of two duplicate sets of unirradiated controls, prior to pooling of both data sets (15 ≤ \( n \) ≤ 20). Integrity indices comprised of averaged peak height ratios for intermediate/short (Index A), long/intermediate (Index B), and long/short loci (Index C), expressed as percentages relative to those for unirradiated controls (indicated by the solid line at 100 %). Reported is the mean ± 95 % confidence interval for integrity indices expressed as the percentage change relative to that for unirradiated controls (indicated by the solid line at 0 %). Statistically significant differences (Wilcoxon-Signed Rank, \( p < 0.05 \)) are denoted by asterisks.

The relative nuDNA integrity indices were compared for hydrated samples relative to those for dehydrated samples (Figure 3.6). The relative integrity indices were significantly lower for hydrated samples after 25 kGy for Index A (18.1 ± 6.43 %, \( p < 0.000 \)), 5 kGy for Index B (16.8 ± 4.18 % lower, \( p < 0.000 \)), and 10 kGy for Index C (18.2 ± 6.84 % lower, \( p < 0.000 \)). No differences were significant at lower doses. Relative integrity indices of hydrated samples were significantly lower than dehydrated samples by up to 48.8 ± 4.43 % (Index A, \( p < 0.000 \)), 67.5 ± 5.52 % (Index B, \( p < 0.000 \)), and 82.2 ± 3.38 % (Index C, \( p < 0.000 \)) at 50 kGy.
Figure 3.6: Change in nuclear DNA dose-response of hydrated samples relative to dehydrated samples. Gamma-irradiation of dried (dehydrated) and liquid (hydrated) whole blood was performed to doses of 1, 5, 10, 25 and 50 kilogray (kGy). Samples from up to 10 individuals were genotyped using the GlobalFiler PCR Amplification Kit for comparison with each of two duplicate sets of unirradiated controls, prior to pooling of both data sets (15 ≤ n ≤ 20). Integrity indices comprised of averaged peak height ratios for intermediate/short (Index A), long/intermediate (Index B), and long/short loci (Index C), expressed as percentages relative to those for unirradiated controls (indicated by the solid line at 100 %). Reported is the mean ± 95 % confidence interval of hydrated sample integrity indices relative to that for unirradiated controls, expressed as a percentage change relative to that for dehydrated samples (indicated by the solid line at 0 %). Statistically significant differences (Mann-Whitney U, p < 0.05) are denoted by asterisks.

3.3.5 Effect of gamma-irradiation on mtDNA integrity

Integrity indices for mtDNA (Section 3.2.7) were expressed after irradiation as a percentage relative to those for unirradiated controls (*relative integrity index*; Figure 3.7). Each integrity index was reduced by irradiation for more cases than they were increased. Increases over all integrity indices were observed in 49 % of dehydrated samples at 1 kGy, 29 % at 5 kGy, 12 % at 10 kGy, 2 % at 25 kGy, and no cases at 50 kGy. Hydrated samples increased in integrity index in 44 % of cases at 1 kGy, but declined by a greater extent as dose increased, compared to dehydrated samples, so that only 6 % of samples increased at 5 kGy and no samples increased after 10 kGy.
Figure 3.7: Mitochondrial DNA (mtDNA) integrity indices as a percentage relative to those for unirradiated controls at each dose. Gamma-irradiation of dried (dehydrated) and liquid (hydrated) whole blood was performed to doses of 1, 5, 10, 25 and 50 kilogray (kGy). Samples from up to 10 individuals were quantified at three mtDNA amplicons (86, 190 and 452 base pairs) by separate SYBR Green assays for comparison with each of two duplicate sets of unirradiated controls, prior to pooling of both data sets (16 ≤ n ≤ 18). Integrity indices were determined from the quantity ratios of targets, 190/86 (Index A), 452/190 (Index B), and 452/86 (Index C), expressed as percentages relative to those for unirradiated controls (indicated by the solid line at 100 %). Extreme values are 1.5× (points) and 3× (asterisks) the interquartile range beyond the first or third quartile and are labelled with the corresponding donor number.

The corresponding average (± 95% confidence interval) change in each integrity index relative to unirradiated controls demonstrated a significant decrease in most cases, without any significant increases (Figure 3.8). For dehydrated samples, significant relative decreases in integrity index from controls occurred after 25 kGy for Index A (13.9 ± 4.74 %, p = 0.003), 10 kGy for Index B (12.1 ± 7.34 %, p = 0.022), and 5 kGy for Index C (15.7 ± 7.86 %, p = 0.010), which were further reduced at 50 kGy to losses of 26.1 ± 4.06 % (Index A, p = 0.003), 59.1 ± 3.55 % (Index B, p = 0.010), and 70.5 ± 3.39 % (Index C, p = 0.011). A relative decrease of each hydrated sample integrity index from controls was significant after only 5 kGy, due to declines of 20.3 ± 5.48 % (Index A, p = 0.003), 24.2 ± 6.99 % (Index B, p = 0.003), and 37.8 ± 8.31 % (Index C, p = 0.003), which were reduced by 74.4 ± 2.43 % (Index A, p = 0.003), 86.1 ± 2.22 % (Index B, p = 0.003), and 96.3 ± 0.734 % (Index C, p = 0.003) at 50 kGy.
Figure 3.8: Change in mitochondrial DNA (mtDNA) integrity indices relative to those for unirradiated controls at each dose. Gamma-irradiation of dried (dehydrated: left) and liquid (hydrated: right) whole blood was performed to doses of 1, 5, 10, 25 and 50 kilogram (kGy). Samples from up to 10 individuals were quantified at three mtDNA amplicons (86, 190 and 452 base pairs) by separate SYBR Green assays for comparison with each of two duplicate sets of unirradiated controls, prior to pooling of both data sets (14 ≤ n ≤ 18). Integrity indices were determined from the quantity ratios of targets, 190/86 (Index A), 452/190 (Index B), and 452/86 (Index C). Reported is the mean ± 95 % confidence interval for integrity indices expressed as the percentage change relative to that for unirradiated controls (indicated by the solid line at 0 %). Statistically significant differences (Wilcoxon-Signed Rank, p < 0.05) are denoted by asterisks.

The relative mtDNA integrity indices of hydrated samples were compared relative to those for dehydrated samples (Figure 3.9). No differences were significant at 1 kGy, while hydrated samples produced significantly lower relative integrity indices after 5 kGy for each index: by 12.1 ± 9.55 % (Index A, p = 0.010), 17.7 ± 8.84 % (Index B, p = 0.020), and 25.3 ± 11.5 % (Index C, p = 0.001). The differences due to sample hydration increased with dose, so that hydrated samples were lower than dehydrated samples at 50 kGy by 65.0 ± 3.64 % (Index A, p < 0.000), 62.5 ± 7.69 % (Index B, p < 0.000), and 86.7 ± 3.52 % (Index C, p < 0.000).
Figure 3.9: Change in mitochondrial DNA (mtDNA) dose-response of hydrated samples relative to dehydrated samples. Gamma-irradiation of dried (dehydrated) and liquid (hydrated) whole blood was performed to doses of 1, 5, 10, 25 and 50 kilogray (kGy). Samples from up to 10 individuals were quantified at three mtDNA amplicons (86, 190 and 452 base pairs) by separate SYBR Green assays for comparison with each of two duplicate sets of unirradiated controls, prior to pooling of both data sets (14 ≤ n ≤ 18). Integrity indices were determined from the quantity ratios of targets, 190/86 (Index A), 452/190 (Index B), and 452/86 (Index C). Reported is the mean ± 95 % confidence interval of hydrated sample integrity indices relative to that for unirradiated controls, expressed as a percentage change relative to that for dehydrated samples (indicated by the solid line at 0 %). Statistically significant differences (Mann-Whitney U, p < 0.05) are denoted by asterisks.

3.3.6 Comparison of nuDNA and mtDNA degradation

The mtDNA integrity indices, expressed as a percentage relative to those for unirradiated controls (‘relative integrity index’), were compared with those for nuDNA at each dose (Figure 3.10). Average relative integrity indices (± 95 % confidence interval) of dehydrated samples were significantly lower for mtDNA than nuDNA after 10 kGy for Index B (17.4 ± 7.03 %, p = 0.006) and 1 kGy for Index C (11.0 ± 8.94 %, p = 0.043), but did not differ significantly at any dose for Index A. Hydrated mtDNA sample integrity was significantly lower than nuDNA integrity after 1 kGy for both Index A (11.8 ± 9.41 %, p = 0.025) and Index C (15.3 ± 8.54 %, p = 0.006), and after 5 kGy for Index B (14.7 ± 10.5 %, p = 0.033). These differences generally increased with dose, leading to significantly lower mtDNA relative integrity indices than those for nuDNA at 50 kGy, by up to 46.8 ± 5.94 % (Index B, p = 0.006) and 55.2 ± 4.26 % (Index C, p = 0.004) for dehydrated samples, and 37.4 ± 7.25 % (Index A, p = 0.003), 41.8 ± 10.5 % (Index B, p = 0.006) and 69.5 ± 6.46 % (Index C, p = 0.003) for hydrated samples.
Figure 3.10: Change in dose-response of mitochondrial DNA (mtDNA) relative to nuclear DNA (nuDNA). Gamma-irradiation of dried (dehydrated: left) and liquid (hydrated: right) whole blood was performed at doses of 1, 5, 10, 25 and 50 kilogram (kGy). Samples from up to 10 individuals were genotyped using the GlobalFiler PCR Amplification Kit, which were also quantified at three mtDNA targets by separate SYBR Green real-time PCR assays, for comparison with each of two duplicate sets of unirradiated controls, prior to pooling of both data sets (13 ≤ n ≤ 20). Integrity indices constituted peak height (nuDNA) or quantity (mtDNA) ratios of intermediate/short (Index A), long/intermediate (Index B), and long/short loci (Index C). Reported is the mean ± 95 % confidence interval of mtDNA integrity indices relative to that for unirradiated controls, expressed as a percentage change relative to that for nuDNA (indicated by the solid line at 0 %). Statistically significant differences (Wilcoxon-Signed Rank, p < 0.05) are denoted by asterisks.

3.4 Discussion

Forensic DNA evidence may be exposed to gamma-radiation in the event of a radiological crime, or during decontamination of biological agents from forensic evidence in cases concerning biosecurity. The doses received by a forensic sample in such cases may be severe. An unshielded Category 1 $^{60}$Co gamma-emitter, removed from a teletherapy unit with typical activity of 150 TBq (IAEA 2005), would exhibit a dose rate approximating 46 Gy/hour at a distance of 1 m or 4.6 kGy/hour at 0.1 m. Doses of gamma-radiation necessary for biological agent decontamination may be upwards of 10 kGy (Dutra et al. 2016; Elliott et al. 1982; Ortatatli et al. 2018). The effect of gamma-irradiation on whole blood exposed to a $^{60}$Co source, with doses ranging from 1 to 50 kGy, was examined. Dried (dehydrated) blood samples were selected for forensic relevance, while liquid (hydrated) blood samples were included to preserve
cell integrity and water content prior to irradiation. The inclusion of both dried and liquid samples enabled contributions to DNA damage from indirect mechanisms (e.g. ROS induction) to be evaluated. Localised sample heating during irradiation may also contribute to this damage (discussed in Section 6.3), but could be expected to mimic a real-world scenario.

These experiments were designed to imitate a scenario where biological evidence is continuously exposed to gamma-radiation for up to near 24 hours (achieving a dose of 50 kGy), before collection and flash freezing. This is representative of the ideal procedure for evidence recovery in a radiation incident; once removed from the radiation source, biological samples should be rapidly frozen, if they cannot be extracted immediately, in order to halt post-irradiation cellular effects. However, delays could occur due to lengthy scene and exhibit processing prior to sample collection. In such cases, continued ROS generation or cell death mechanisms initiated by irradiation can contribute to greater levels of DNA degradation, at least in live cells at doses well below 1 kGy (Hu et al. 2017; Joseph et al. 2014; Yamaguchi & Kashiwakura 2013). Gamma-irradiation beyond 1 kGy was applied by Abbondante (2009) to examine the effects of time-to-analysis on various dried sample types over a period of four weeks. This revealed inherent analytical variation of STR genotypes to be greater than any effect of delayed analysis, although the sample storage conditions during the investigated time periods were not detailed. These results may suggest that higher doses do not permit the same continuous adverse cellular effects as lower doses, or instead, reflect the dried cellular preparations used in their study, preventing any significant damaging activity post-irradiation. A time-to-analysis study may likely have more relevance to hydrated sample types (discussed in Section 6.3).

In this current study, samples were flash frozen post-irradiation to eliminate the possibility of any delayed effects, representing a ‘best case’ evidence recovery scenario, and to evaluate only the immediate radiation-induced effects upon analysis. Genotypes at forensic autosomal STR loci did not show signs of degradation pre-irradiation, but did exhibit a high frequency of samples with heterozygote peak imbalances (Figure 3.1). Doses above 10 and 25 kGy for hydrated and dehydrated samples, respectively, produced greater frequencies of imbalances than the unirradiated controls, which increased progressively up to 50 kGy (Figure 3.2). The level of imbalance was consistent with a significant reduction in overall peak heights for hydrated and dehydrated samples after respective doses of 25 and 50 kGy (Figure 3.3A). This was associated with a typical degradation pattern, whereby a progressive decline in peak height occurred as amplicon size increased. However, the impact on genotype reporting based on peak
height and heterozygote peak imbalance thresholds was minor, affecting a maximum of six loci due to imbalance, while no loci dropped below peak amplitude thresholds except for larger loci (> 225 bp) of hydrated samples at 50 kGy. Genotyping thresholds, which are routinely implemented to ensure the fidelity of forensic genotypes, were suitable to detect genotype non-concordance due to dropout of a single heterozygous allele, which affected one-fifth of subthreshold loci. While stochastic thresholds can be lowered to reduce dropout incidence, this cannot be recommended without more comprehensive validation of such thresholds due to the possibility of miscalling alleles.

The resilience of STR profiles against high doses of gamma-radiation is consistent with previous studies. Hoile et al. (2010) described unpublished results, from the dissertation by Abbondante (2009), whereby full AmpF/STR Profiler Plus (Applied Biosystems) genotypes generated from dried blood were attainable up to 50 kGy, with a reduction in peak heights after 10 kGy. Similarly, Neureuther et al. (2014) found that PowerPlex ESX 17 System (Promega) STR profiles did not differ significantly between untreated and gamma-irradiated dried saliva and blood samples at 25 kGy. Hodgson and Baxter (2013) made similar observations using AmpF/STR Identifiler (Applied Biosystems), with pre-irradiation degradation of their aqueous buccal cell suspensions contributing more to allele dropout. Monson et al. (2018) found full AmpF/STR Identifiler Plus (Applied Biosystems) profiles from dried bloodstains were attainable up to 90 kGy of gamma-radiation. Shaw et al. (2008) demonstrated greater levels of degradation at 56.4 kGy, where only 40 % of dried saliva samples produced full AmpF/STR SGM Plus (Applied Biosystems) profiles, with half of the remaining partial profiles comprising less than four allele designations. In comparison to electron beam (beta) radiation, which is more common for biological decontamination, Shaw et al. (2008) found only 70 % of dried saliva samples to produce full profiles after 50 kGy; however, this was an improvement to their gamma-radiation results. Withrow et al. (2003) attained full Profiler Plus and Cofiler (Applied Biosystems) profiles from electron beam-irradiated saliva (from envelopes) up to 51.6 kGy, for which HV1 sequencing was also successful. However, post-irradiation sample storage conditions could be a significant point of difference between these studies. Further, these studies did not report on heterozygote allele imbalance.

Heterozygote imbalances are typical of PCR inhibition or degradation, particularly of longer targets (Hansson et al. 2017; Thompson et al. 2014). Given the imbalances present in the unirradiated sample controls (Figure 3.1), PCR inhibition from the carryover of haem/haematin
and/or EDTA from the blood collection and extraction process was likely to have occurred. Although the qPCR could not confirm the presence of inhibitors (Section 3.3.1), lending some doubt to this hypothesis, it is possible that differences in assay chemistries and length of targets could alter the sensitivity of qPCR and STR assays to inhibition (Hall et al. 2013; Opel et al. 2010; Wang et al. 2008). In comparison to the unirradiated controls, the frequency of heterozygote peak imbalance was lessened at doses of up to 10 and 25 kGy for hydrated and dehydrated samples, respectively (Figure 3.2). Overall peak heights were significantly increased from that of unirradiated controls at and below doses of 5 kGy (hydrated) and 10 kGy (dehydrated), with approximately equivalent overall peak heights for irradiated and unirradiated samples at these doses. It was not thought that inherent template damage or DNA degradation of the controls was responsible for these observations, as this would only be aggravated by irradiation, rather than alleviated, considering that the doses applied are beyond those expected to initiate any adaptive DNA repair response (Section 1.3.3).

An explanation for these improvements to STR genotypes at lower doses is offered by the possible degradation of PCR inhibitors, which may occur more readily after gamma-irradiation than that of DNA. Indeed, inhibitors such as haematin and EDTA are degraded by gamma-radiation exposures at doses below 1 and 5 kGy, respectively (Jung et al. 2004; Rafiei et al. 2017). It is then possible that more substantial increases in peak height after irradiation, which was observed for TH01, D2S1338 and DYS39 (Figure 3.3A), is due to an inherent susceptibility of certain amplicons to inhibition previous to irradiation. These inhibitors may be less easily recovered into the extraction medium after drying, along with DNA (Section 3.3.1), due to the need for additional transfer steps that are not required for liquid samples. This is consistent with the lower levels of peak imbalance and slightly higher overall peak heights of dehydrated controls, and the more frequent peak height increases observed at initial doses of hydrated samples (Figure 3.3B). These findings suggest potential for such lower doses of gamma-radiation to improve genotypes of inhibited samples, a common occurrence for forensic specimens. However, verification of this hypothesis is required (discussed in Section 6.3).

Integrity indices similarly conformed with the improvements observed for STRs, where an increase in integrity index from unirradiated controls is accounted for by a greater recovery of the longer amplicon after irradiation, as compared to the shorter amplicon. While inhibition could be expected to impact all target lengths, longer targets are more susceptible (Opel et al. 2010; Wang et al. 2008) and therefore more greatly alleviated by possible inhibitor removal.
after irradiation. Integrity indices for nuDNA more frequently and significantly increased, relative to those for unirradiated controls, for most indices at 1 kGy for both hydrated and dehydrated samples and up to 5 kGy for dehydrated samples only (Figure 3.4 and Figure 3.5). Size-matched mtDNA integrity indices were less frequently and not significantly increased from those of unirradiated controls (Figure 3.7 and Figure 3.8). The observation of increased mtDNA integrity indices after irradiation may imply an inhibitor interaction similar to that observed for nuDNA, although the lower frequency of such cases and overall nonsignificance may suggest some change in the inhibitor effect. This disparity between nuDNA and mtDNA could be explained by differences in the interaction of inhibitors with (a) nuDNA versus mtDNA targets, such as from differences in structural conformation or binding regions (for template-binding inhibitors); and/or (b) PCR chemistries, which is evidenced by the portrayal of inhibition in STR genotypes without inhibited nuDNA or mtDNA qPCRs (Section 3.3.1).

As dose increased, DNA degradation outweighed the ‘signal boost’ from possible inhibitor decomposition, resulting in a reduction in nuDNA and mtDNA integrity indices. Sensitivity to degradation proceeded, generally, in order of Index A < Index B < Index C, consistent with increased susceptibility of amplicons to degradation with length. Doses of 10 and 25 kGy were sufficient to cause a significant loss of hydrated and dehydrated nuDNA integrity, respectively (Figure 3.5). Only 5 kGy was required to significantly reduce mtDNA integrity of hydrated samples, with up to 25 kGy required for dehydrated samples (Figure 3.8). A radioprotective effect was therefore conferred by cellular desiccation (Figure 3.6 and Figure 3.9). While dried biological stains are common forensic specimens, damage intermediate to that of dried and aqueous samples could be expected for samples irradiated over the course of drying. While ROS interactions may not be completely removed by sample dehydration, owing to the possible persistence of residual moisture, this result highlights a greater propensity for ROS generation from water radiolysis, or other cellular interaction. This would account for the increased DNA damage when cell hydration is completely preserved.

This effect also contributed to a greater extent of mtDNA damage as compared to nuDNA (Figure 3.10). This is not unexpected, given the known influence of ROS mediators on mtDNA damage, produced from mitochondrial hyperfunction after ionising-irradiation, coupled to a reduced DNA repair capacity within the mitochondria (Leach et al. 2001; Yakes & van Houten 1997; Yamamori et al. 2012). While such cell responses may be precluded by the high dose rate (2 kGy/hour) and total dose (1-50 kGy) received, which could be expected to rapidly terminate
cell function, upregulation of mitochondrial ROS may be possible from the induction of cell death pathways, such as apoptosis or a form of necrosis (Golstein & Kroemer 2006; Kim et al. 2008). However, such cell death studies include dose regimens well below those applied here.

These modes of additional damage to mtDNA rely on the cell maintaining functional integrity prior to irradiation. A negligible difference between nuDNA and mtDNA extracted from desiccated cells should then be expected at every dose, which was only true for Index A. This may implicate residual moisture to preserve some level of cell integrity and thus a restricted, rather than removed, capacity for mitochondrial ROS formation. This observation could also be attributed to radioprotection afforded by the structural arrangement of nuDNA into compact chromatin (i.e. heterochromatin) and association with histone proteins (Falk et al. 2008; Takata et al. 2013). However, histones do not protect DNA from electron transfer reactions and can enhance DNA damage under certain conditions (Cullis et al. 1987; Liang & Dedon 2001). Further, nucleoid compaction of mtDNA by association with histone-like DNA-binding proteins (e.g. mitochondrial transcription factor A, TFAM) confers an almost equivalent level of protection to that of histones (Gouliaeva et al. 2006). These considerations are reviewed by Alexeyev et al. (2013). Thus, current understanding of the structural arrangement of nuDNA versus mtDNA may not explain the observed differences in DNA damage. Ionisation events may also occur with greater frequency within the mitochondria than nucleus when mitochondrial volume exceeds 20 % of the total cell volume (Kam et al. 2013). This effect would synergise with an increase in cellular mitochondrial content following ionising-irradiation (Yamamori et al. 2012).

The drying of blood into solid flakes could also be considered to act as a physical barrier to radiation. However, Hall et al. (2014) compared dehydrated and suspended cell-free DNA, finding dried bloodstains to be more sensitive to STR genotyping failure after exposure to UV-B radiation. While nonionising, this observation demonstrates that dried blood layers do not prevent electromagnetic waves from interacting with blood cells and supports a mechanism for DNA damage from a level of continued activity in desiccated cells. Furthermore, the high energy gamma-ray photons are highly penetrating (far more than UV photons), requiring material of appropriate density and thickness to significantly impact their attenuation through matter (Waly et al. 2016). For this reason, a negligible difference in absorbed dose was also considered for liquid and dried samples due to their respective preparation into glass vials and under glass coverslips (discussed in Section 6.3).
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The mtDNA targets evaluated for degradation, although of similar length to amplicons applied for mitochondrial HVR sequencing, are not direct indicators of HVR sequencing success. This is because of their location within the 12S and 16S rRNA coding regions, rather than the non-coding control region (D-loop) itself, where the HVRs are located (Section 2.1). These targets were selected based on their potential amenability to multiplexing, considering homology of sites for hydrolysis probe binding that cannot be achieved in the control region (Section 6.2). However, Zhou et al. (2012) identified the D-loop as being the most susceptible mtDNA region to X-ray-induced oxidative damage at low doses (up to 8 Gy), and therefore the level of damage to HVRs could be expected to be greater than the rRNA coding regions. Monson et al. (2018) demonstrated no loss of sequencing fidelity for both HV1 and HV2 of single hair fibres after a dose of 90 kGy of gamma-radiation, which was consistent with STR genotyping success of dried blood, although these results were not directly comparable due to the use of non-equivalent cellular material. Further evaluation of damage to HVR sequences relative to mtDNA integrity indices is required at such high doses that are necessary to degrade the forensic STRs.

3.5 Summary and conclusions

Gamma-irradiation was shown to elicit greater damage to mtDNA than nuDNA at equivalent doses, which was more substantial in whole blood samples without desiccation. This is likely to implicate ROS induction from water radiolysis and mitochondrial function (such as during the onset of cell death) upon DNA damage when sample integrity and water content were initially preserved. Consequently, a radioprotective effect of sample dehydration, as is commonplace for forensic biological specimens, was found. However, disparity between nuDNA and mtDNA integrity in dried samples suggests that additional radioprotection is afforded to nuDNA, possibly due to structural arrangement, fewer nuclear ionisation events, and/or the presence of residual moisture that preserves some cell functionality to mediate a limited mitochondrial ROS response. This requires further investigation.

DNA degradation, as revealed by integrity indices, did not substantially impact forensic STR genotyping capability, especially of the more forensically relevant dried samples. In fact, lower doses of gamma-radiation were beneficial to nuDNA integrity and STR genotypes, both increasing overall peak height and reducing the rate of heterozygote imbalance as compared to
that of unirradiated controls. It is possible that this was attributable to the preferential degradation of low levels of PCR inhibitors, another common occurrence within forensic samples, and warrants further investigation.

At higher doses, while signs of degradation (tailing of longer loci) were apparent, full profiles were possible across the entire dose range for both sample preparations. Heterozygote imbalances contributed to most cases of subthreshold alleles, rather than peak height dropout, which was only encountered for hydrated samples after 50 kGy. Precautions that are routinely employed to ensure the fidelity of forensic genotypes (*i.e.* genotyping thresholds) were sufficient to detect genotype non-concordance at this high dose; however, a more comprehensive analysis of such thresholds is still required for gamma-irradiated samples, particularly upwards of 50 kGy. Thus, at the doses examined, STR genotyping capability was not greatly impacted by gamma-irradiation, even in the possible presence of low-level inhibition. This may preclude the need for mtDNA as an identification tool in such cases, at least where gamma-radiation is the only genetic insult.
Chapter 4: Development of methodology for the quantitative analysis of cholesterol oxidation biomarkers and DNA co-extraction
4.1 Introduction

COPs are a class of lipids derived from cholesterol oxidation, which are generated via the action of metabolic enzymes, commonly P450 cytochromes, and ROS under conditions of oxidative cellular stress (Section 1.3.2.2.1). These compounds have been implicated in various disease states, particularly those that involve such oxidative stress (Olkkonen et al. 2012; Poli et al. 2013; Sabuncuoğlu & Öztas 2014; Vejux et al. 2008). The hyperproduction of ROS is also a major pathway for ionising radiation-induced biological damage (Azzam et al. 2012; Leach et al. 2001; Yamamori et al. 2012). Accordingly, formation of various COPs has been observed after accidental gamma-radiation exposures (Bertho et al. 2009; Bertho et al. 2008). Markers of DNA oxidative damage have also been monitored under conditions of oxidative stress, including after ionising-irradiation (Dincer et al. 2007; Mecocci et al. 1994; Shen et al. 2007; Wilson et al. 1993; Wu et al. 2004).

While DNA integrity is the classical paradigm for cell viability (Iyer & Lehnert 2000; Munro 1970a; Munro 1970b; Radford 1986), a role for COPs and other cellular lipids in the processes leading to cell survival and death is now well-established. These compounds are indicative of both metabolic and ROS-mediated cellular change (Benderitter et al. 2003; Haimovitz-Friedman et al. 1994; Kiang et al. 2012; Krisko et al. 2012; Mishra 2004; Noguchi et al. 2014; Nury et al. 2013; Schneiderman et al. 2001). Thus, a method for the simultaneous extraction and analysis of lipid and DNA biomarkers may be of value to studies of oxidative stress related pathogenesis, as well as studies of cell damage and survival/death mechanisms in general.

Co-extraction of both DNA and total lipids from soil microorganisms was conducted by Kehrmeyer et al. (1996). Their method was an adaptation of the Bligh and Dyer (1959) lipid extraction solvent system, involving homogenisation of aqueous sample in three times the volume of chloroform:methanol (1:2 v/v), after which chloroform:water (1:1 v/v) was added to achieve a phase separation (Section 1.4.1). The original Bligh and Dyer technique was developed for extraction of lipids from fish muscle and is itself a modification of the method by Folch et al. (1957). The original method employed a larger solvent:sample ratio (20:1 v/v) and application of chloroform:methanol at 2:1 (v/v), which is regarded as being most efficient for maximal recovery and diverse sample types (Axelsson & Gentili 2014; de Morais et al. 2010; Iverson et al. 2001; Reis et al. 2013). Both methods isolate total lipids into a lower organic phase alongside extraction of hydrophilic contaminants, inclusive of DNA, into an upper
aqueous phase. Kehrmeyer et al. (1996) used this system to collect and ethanol precipitate DNA from the aqueous phase, allowing the organic phase to be processed for selected lipids.

Purification of COPs and, if desired, F\textsubscript{2}-isoprostanes, a reliable marker of lipid peroxidation, can be achieved simultaneously with the saponification and SPE detailed by Lee et al. (2008b). This procedure was originally developed for direct saponification of plasma samples, with the intention to analyse oxygenated lipid markers by GC-MS. After evaluation of the literature (Section 1.4.4), GC-MS was considered to offer the best approach for the sensitive measurement of COPs. Lee et al. (2008b) used a mixed-phase resin for the SPE containing hydrophilic and hydrophobic regions that retained neutral and slightly polar COPs, whilst a quaternary ammonium anion exchanger retained the acidic F\textsubscript{2}-isoprostanes (Section 1.4.2.2). Hydrophilic contaminants are washed away by an aqueous solution of ammonium hydroxide that retains neutral/hydrophobic sterols and prostanoids under high pH. A second wash with methanolic formic acid may protonate basic compounds and free fatty acids for their removal. Neutral and polar interactions are disrupted by the use of nonpolar (hexane) and polar (ethyl acetate) solvents for sterol elution. The remaining acidic prostanoids are eluted by a pH adjustment with acetic acid to protonate the carboxylate anion (COO\textsuperscript{-}) and further hydrophilic/phobic interactions prevented with a combination of nonpolar (hexane) and polar (ethanol) solvents.

The primary problem with COPs analysis is cholesterol autoxidation during sample workup, leading to spurious COP formation (Section 1.4.5). Inclusion of antioxidants is one means by which the generation of autoxidation artefacts can be prevented (Busch & King 2009). The lipid-soluble antioxidant, BHT, is regularly employed in this regard (Griffiths et al. 2013; Lee et al. 2008b; Pataj et al. 2016). Derivatisation of the active hydroxyl hydrogen atoms to nonreactive groups is another means to prevent artefact formation, specifically from thermal decomposition during sample analysis, which is a requirement for analysis by GC-MS (Section 1.4.3). This is commonly achieved by silylation of the hydroxyl groups to TMS ethers.

The present study aimed to develop a GC-MS analysis method for the quantification of various COPs, as well as a solvent system for DNA and lipid co-extraction from peripheral blood mononuclear cells (PBMCs). COPs were selected to include an array of ring-oxidised COPs that represent autoxidation as the exclusive or predominant generation pathway (7\textalpha-HC, 7\bet-HC and 7-KC), as well as several tail-oxidised COPs that represent exclusive or primarily metabolic (enzymatically-derived) formation (24-HC, 25-HC and 27-HC). The cholesterol precursor,
lathosterol, was also selected for analysis as an indicator of cholesterol biosynthesis. The structures and functions of these sterols are detailed in Section 1.3.2.2.1. The internal standard for quantification was 19-HC due to its structural and biochemical similarity to other COPs and absence from the biological matrix. A recovery standard, betulin, was applied in order to determine internal standard recoveries. The requirements of such standards are detailed in Section 1.4.4.5. The method development included an evaluation of purification approaches for the isolation of DNA and COPs by ethanol precipitation and SPE, respectively. The developed procedure would be applied for the analysis of COPs after gamma-irradiation of human cells (Chapter 5).

4.2 Methodology

4.2.1 Experimental design

The method developed by Lee et al. (2008b) offered a foundation for COPs analytical method development. Targeted COPs/sterols were 7α-HC, 7β-HC, 7-KC, 24-HC, 25-HC, 27-HC and lathosterol, with 19-HC as the internal standard and betulin as the recovery standard. Method development proceeded, firstly, by optimising and verifying the COP detection and quantification approach by GC-MS. A derivatisation procedure was optimised to produce single peaks corresponding to each derivatised analyte, with baseline resolution achieved via optimisation of a GC temperature program. A large volume injection procedure was developed for the sensitive analysis of trace sterols. For selective detection and quantification of each analyte, a SIM method was developed. Validation of assay linearity, sensitivity, quantification limits, accuracy, precision, specificity and matrix effects was performed from calibration curves.

Secondly, extraction and purification procedures were developed, with the intention to co-extract both DNA and selected sterols from cell substrates for a comparative assessment of DNA integrity and COPs formation after low doses of gamma-radiation. Human PBMCs were expected to contain approximately 0.54 ng of 7α-HC, 0.29 ng of 7β-HC, 2.20 ng of 7-KC, 0.12 ng of 24-HC, 0.11 ng of 25-HC and 0.03 ng of 27-HC per 1×10⁶ cells. These values were estimated from the cholesterol content ratio of PBMCs (2.10 µg/1×10⁶ cells) to erythrocytes (0.13 µg/1×10⁶ cells) (Gottfried 1967), provided the quantities of erythrocyte COPs (Pataj et al. 2004).
and assuming the same relative proportions for PBMCs. Given the expected low occurrence of COPs in the samples under examination, it was critical to ensure their maximal recovery; thus, a conventional lipid solvent extraction system was employed as the basis for extractions, which was modified from Kehrmeyer et al. (1996) and Folch et al. (1957) to facilitate DNA recovery. Total DNA was expected to be in high abundance, ~7 µg per $1 \times 10^6$ cells (Gillooly et al. 2015), although the efficacy of DNA recovery using the lipid extraction system was unknown.

Method development for both analyte classes was accomplished in a series of stages. The solvent extraction was applied in order to inform a cell lysis approach and appropriate aqueous buffer for maximal DNA recovery after ethanol precipitation. The procedure by Lee et al. (2008b) was further implemented as the basis for sterol saponification and isolation by SPE. The impact of aqueous buffer (plasma surrogate) and BHT upon saponification and SPE was evaluated by the recovery of spiked analytes. The volumes of SPE wash and elution solvents were optimised to maximise sterol recoveries. The method was validated by an examination of spiked analyte recoveries from each stage of the analytical process and from the overall procedure. In summary, the following strategy was employed in an attempt to develop a combined DNA and COPs analysis protocol:

1. Develop an analysis protocol for selected sterols by GC-MS, including a:
   a. Derivatisation procedure
   b. Injection method and temperature program
   c. Quantitative SIM method
2. Develop and optimise an analytical procedure for the co-extraction of DNA and lipids, including a:
   a. Solvent system for the co-extraction of cellular DNA and lipids
   b. Saponification and SPE procedure for the isolation of selected sterols
3. Validate the optimised method based on calibration curves and sterol recovery
4.2.2 Chemicals and standards

Sterol standards included lathosterol from Sigma-Aldrich, 7α-HC and 7β-HC from Steraloids (Newport, USA), 7-KC and 27-HC from Avanti Polar Lipids (Alabaster, USA), and 24-HC, 25-HC, 19-HC (internal standard) and betulin (recovery standard) from Sapphire Bioscience (Redfern, Australia). All standards were minimally ≥ 98 % pure and stocks were dissolved in ethyl acetate (HPLC grade; Sigma-Aldrich).

Acetic acid (Scharlau, Barcelona, Spain), chloroform with 1 % ethanol stabiliser (RCI Labscan, Bangkok, Thailand), methanol (Honeywell, Morris Plains, USA), hexane (Scharlau), and pyridine (Sigma-Aldrich) were HPLC grade. Ammonium hydroxide (30 % w/w ammonia basis), formic acid (Sigma-Aldrich), and anhydrous sodium sulphate (Sigma-Aldrich) were ACS reagent grade. Hydrochloric acid (32 % w/w; Chem-Supply, Gillman, Australia) and anhydrous sodium acetate (Fronine, Riverstone, Australia) were AR grade. Methanolic 1 M potassium hydroxide and BSTFA (1 % v/v TMCS) were from Sigma-Aldrich. Glassware silanisation was carried out by rinsing with 5 % dimethyldichlorosilane (DMCS; Sigma-Aldrich) in toluene (AR grade; Ajax Finechem, Taren Point, Australia), followed by rinsing with ethyl acetate, then methanol.

TE buffer (10 mM Tris-chloride, pH 8.0, 0.1 mM EDTA) was prepared from the same chemicals as applied in Section 2.3.1.1, with the same general laboratory preparation for DNA work. Extraction buffers included TE1 buffer (10 mM Tris-chloride, pH 8.0, 1 mM EDTA) and high-salt TE1 buffer, which contained 3 M sodium chloride (Sigma-Aldrich). Phosphate-buffered saline (PBS) was prepared from dissolvable tablets (Amresco, Solon, USA) for 137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer, pH 7.4 ± 0.1. Ethanol precipitation was performed with 200 proof ethanol for molecular biology (Sigma-Aldrich).
4.2.3  Samples

4.2.3.1  Spiked calibration and recovery samples

All spiked samples (for instrument calibration and recovery experiments) were created by adding sterol standards dissolved in methanol into blank, silanised glass Pyrex 9 mL culture tubes with phenolic screw caps (Corning Inc., Corning, New York) for extraction, or reaction vials for direct derivatisation. Mock matrix spiked samples comprised of 5 mL high glucose (D6429) Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich) spiked with 12 µg cholesterol to simulate cell matrix as applied for irradiation experiments (Chapter 5). Blank and DMEM mock matrix samples were subjected to the entire sample workup procedure according to the definitive methods (Section 4.2.4), which took place prior to spiking of sterols pre-derivatisation (calibration curves, Section 4.2.8.1) or at various stages of the analytical method (recovery experiments, Section 4.2.8.3).

4.2.3.2  Peripheral blood mononuclear cells

For experiments involving PBMCs, whole blood was collected with informed consent from a single volunteer by venepuncture into lithium heparin-coated 10 mL Vacutainers (Becton Dickinson). A density gradient centrifugation was carried out to isolate PBMCs using Ficoll-Paque Plus (GE Healthcare Life Sciences, Chicago, USA). Whole blood from a single 10 mL blood tube was diluted 1:1 (v/v) with PBS in a sterile 50 mL tube. Approximately 9 mL diluted whole blood was laid over 3 mL Ficoll-Paque Plus in sterile 15 mL tubes. Density separation was achieved at a relative centrifugal force (RCF) of 650 times gravity (×g) for 30 minutes at 20 ºC in a Centrifuge 5810R (Eppendorf, Hamburg, Germany), utilising swing-buckets with the brake turned off. The buffy coat containing PBMCs was collected into new 15 mL tubes and washed thrice with PBS, pelleting cells at 300×g for 15 minutes at 20 ºC and decanting the supernatant each time. Before the third wash, PBMC pellets were resuspended in 0.5 mL PBS and pooled into a single 15 mL tube. The final PBMC pellet was resuspended in 0.5 mL DMEM.
4.2.4 Definitive methods

4.2.4.1 Solvent extraction

Frozen samples were freeze-dried in a FreeZone Plus 6 Litre Cascade Console Freeze Dry System 7934001 (Labconco, Kansas City, USA) overnight before extraction. The vacuum chamber was connected to an ultrapure nitrogen supply (99.999 %) for venting and during loading and unloading of samples. Immediately after freeze-drying, a volume of 0.67 mL methanol (containing 50 µg BHT) was added to the lyophilised sample, followed by 0.1 mL TE\textsubscript{1} buffer, with vortexing for 15 seconds. A volume of 1.33 mL chloroform was added, then the samples were vortexed and allowed to continue mixing on an RM2 Medium Reciprocating Shaker (Ratek, Boronia, Australia) for 20 minutes at 200 strokes per minute. A biphasic system was formed by the addition of 0.1 mL TE\textsubscript{1} buffer, and mixing was continued for 10 minutes. Phase separation was achieved by centrifugation at 3000\texttimes g for 2 minutes at 4 °C.

The upper (aqueous) phase was collected into sterile 1.5 mL tubes for DNA processing (Section 4.2.4.2.1). The lower (organic) phase was passed through a glass transfer pipette ‘plugged’ with anhydrous sodium sulphate (pre-rinsed with 2:1 v/v chloroform:methanol) to remove residual moisture and minimise lipid contamination before collection into clean silanised Pyrex 9 mL tubes. The plugged pipette was post-rinsed with 0.5 mL chloroform:methanol (2:1 v/v) and the flow-through additionally collected. The collected organic phase was evaporated under a stream of ultrapure nitrogen in a 24 Position N-EVAP Nitrogen Evaporator (Organomation, Berlin, USA) before purification of selected sterols (Section 4.2.4.2.2).

4.2.4.2 Purification of DNA and sterol extracts

4.2.4.2.1 Ethanol precipitation of DNA

The aqueous phase collected from the solvent extraction (Section 4.2.4.1) was processed by ethanol precipitation to isolate DNA. For every 1 volume of aqueous phase, 0.1 volumes sodium acetate (3 M, pH 5.2) and 3 volumes cold (-20 °C) absolute ethanol were added. Samples were mixed by inversion and incubated at -20 °C for 15 minutes before pelleting the DNA at 12,000\texttimes g for 45 minutes in a Heraeus Pico 21 Microcentrifuge (Thermo Scientific, Waltham, USA). The supernatant was decanted, and the DNA pellet washed with 200 µL cold
(-20 °C) 70 % (v/v) ethanol by inverting to mix and incubating at -20 °C for 15 minutes. The DNA was pelleted again at 12,000×g for 15 minutes and the 70 % ethanol wash repeated to remove excess salts. The centrifugation was repeated once more and the supernatant decanted. The final DNA pellet was air-dried and resuspended in 20 μL TE buffer.

4.2.4.2.2 Saponification and SPE of sterols

The dried organic residue collected from the solvent extraction (Section 4.2.4.1) was reconstituted in 1 mL methanolic potassium hydroxide for alkaline hydrolysis of esterified fatty acids. Samples were purged with ultrapure nitrogen before incubation at 37 °C for 30 minutes. After saponification, 1 mL methanol, 0.2 mL hydrochloric acid (5 M), then 2.5 mL formic acid (20 mM, pH 4.5) were added and the mixture gently vortexed.

SPE was performed with Oasis MAX 3 cc Vac Cartridges (Waters, Milford, USA) containing 60 mg sorbent of 30 µm particle size and an Alltech 12-Port Vacuum Manifold (Grace, Columbia, USA). The cartridges were washed with 2 mL methanol, then preconditioned with 2 mL formic acid (20 mM, pH 4.5), prior to loading of the hydrolysed sample. The loaded cartridges were washed with 2 mL ammonium hydroxide (2 % v/v), then 1.8 mL methanol:20 mM formic acid, pH 4.5 (40:60 v/v). Sterols were eluted with two combined fractions of 2 mL hexane, then 2.5 mL hexane:ethyl acetate (70:30 v/v) into 5 mL silanised glass vials. The elution solvents were evaporated under nitrogen stream, then reconstituted in 0.5 mL ethyl acetate for transfer into silanised 1 mL Reacti-Vial Small Glass Reaction Vials (Thermo Scientific) and the solvent evaporation repeated.

4.2.4.3 Derivatisation

Dried sterols were redissolved into 200 μL pyridine and 200 μL BSTFA (1 % v/v TMCS). Samples were vortexed and incubated at 80 °C for 2 hours in a dry block heater with intermittent vortex mixing every 30 minutes. After derivatisation to TMS ethers was complete, the solvents/reagents were evaporated under nitrogen stream and the dried silylated sterols were reconstituted in 30 μL ethyl acetate containing 40 % (v/v) BSTFA (1 % TMCS) in silanised glass GC vial inserts. Both pyridine and ethyl acetate were ‘dried’ by passing aliquots of each solvent through a transfer pipette plugged with anhydrous sodium sulphate prior to use in order to remove residual moisture.
4.2.4.4 GC-MS analysis

Quantitative analysis of selected COPs and lathosterol was performed using a 7890A GC System coupled to a 5975C inert Mass Selective Detector (MSD), equipped with a 7693 Autosampler and MSD Chemstation G1701EA, version E.02.00.493 (Agilent Technologies, Santa Clara, USA). The MSD consisted of a 70 eV EI source at 230 °C and quadrupole mass analyser at 150 °C, with detection of fragment ions via a triple axis high-energy dynode coupled electron multiplier. The GC inlet and GC to MS transfer line were maintained at 280 °C. The carrier gas was ultrapure helium (99.999 %) with a flow rate of 0.8 mL/min.

Multiple large volume splitless injections (2 × 5 µL) were made slowly (1 µL/s), with a 2 second viscosity and 30 second injection delay, into a 280 °C inlet containing a 4.0 mm (internal diameter) split/splitless liner with single bottom taper. The septum purge flow (3 mL/min) was switched on after 2 minutes, together with the purge flow to the split vent (100 mL/min). Analytes were ‘cold-trapped’ at the start of a SLB-5ms (30 m × 0.25 mm × 0.25 µm; Supelco, Bellefonte, USA) column at 80 °C for 2 minutes. For analyte separation and elution, the temperature was raised to 260 °C at maximum speed (initially 120 °C/min) and held for 4 min, then to 280 °C at 2 °C/min and held for 4 min, then to 290 °C at 2 °C/min without hold, then to 300 °C at 4 °C/min and held for 1.5 min. Mass spectra were collected after a 12 minute solvent delay via SIM operation using the program designed for either blank or DMEM matrix-extracted samples (Table 4.1).

Table 4.1: Selected ion monitoring (SIM) program for sterol/oxysterol trimethylsilyl (TMS) ethers in blank and mock matrix samples.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>SIM window start (min)</th>
<th>Dwell (ms)</th>
<th>Quantifier ion (m/z)</th>
<th>Qualifier ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
<td>Matrix</td>
<td>Blank</td>
<td>Matrix</td>
</tr>
<tr>
<td>7α-HC&lt;sup&gt;a&lt;/sup&gt; diTMS ether</td>
<td>17.00</td>
<td>17.50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>19-HC diTMS ether&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.60</td>
<td>20.20</td>
<td>50</td>
<td>33</td>
</tr>
<tr>
<td>Lathosterol TMS ether</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7β-HC diTMS ether</td>
<td>20.85</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>24-HC diTMS ether</td>
<td>23.20</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>25-HC diTMS ether&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25.50</td>
<td>25.00</td>
<td>50</td>
<td>38</td>
</tr>
<tr>
<td>7-KC&lt;sup&gt;c&lt;/sup&gt; TMS ether</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27-HC diTMS ether</td>
<td>26.80</td>
<td>27.55</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Betulin diTMS ether&lt;sup&gt;d&lt;/sup&gt;</td>
<td>28.25</td>
<td>28.25</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hydroxycholesterol  
<sup>b</sup> Internal standard  
<sup>c</sup> Ketocholesterol  
<sup>d</sup> Recovery standard
4.2.4.5 Quantification

Analyte quantification was achieved from the EIC of the quantifier ion (Section 4.2.4.4) for each analyte based on the preparation of calibration curves (Section 4.2.8.1). Calibration curves were composed of the analyte peak area \( (A_x) \) and concentration \( (C_x) \) relative to those of the internal standard \( (A_y, C_y) \), expressed as common logarithms, \( i.e. \log(A_x/A_y), \log(C_x/C_y) \). This enabled calculation of unknown analyte concentrations (substituting \( C_x \)) from the \( y \)-intercept \( (b) \) and slope \( (m) \) of the calibration line (Equation 4.1).

\[
C_x = C_y \left(10^{-b} \frac{A_x}{A_y}\right)^{\frac{1}{m}} \quad \text{Equation 4.1}
\]

The internal standard (19-HC) recovery was determined from the response factor (ratio of analyte peak area to injected mass) of a recovery standard (betulin) relative to that of the internal standard. The recovery standard was spiked into samples immediately prior to derivatisation at the same mass as the internal standard. The relative response factor (RRF) of the recovery standard was obtained for each injection of calibration samples as applied for the generation of calibration curves. The averaged RRF was employed to calculate the quantity/recovery of internal standard \( (W_y) \) from the recovery standard weight \( (W_z) \) and peak area \( (A_z) \), as well as the internal standard peak area \( (A_y) \) (Equation 4.2).

\[
C_y = \frac{A_y C_z RRF_z}{A_z} \quad \text{Equation 4.2}
\]

4.2.5 Optimisation of GC-MS analytical procedures

The procedure by Lee et al. (2008b) formed the basis of method optimisation for COPs analysis by GC-MS. Instrumentation and general parameters (e.g. flow rate / linear velocity, transfer line and MS temperatures) were unchanged in the definitive method (Section 4.2.4.4). The original procedure involved derivatisation using BSTFA (1 % v/v TMCS) with equal volume pyridine at room temperature for 2 hours, then injection of 2 µL sample onto a BPX5 (12 m × 0.22 mm × 0.25 µm; SGE Analytical Science, Ringwood, Australia) column held at 160 °C for 1 minute, then increased at 40 °C/min to 300 °C for 6 minutes. Ethyl acetate was used as injection solvent.
instead of undecane, which was used by Lee et al. (2008b). The derivatisation method (Section 4.2.5.1) and instrument parameters (Section 4.2.5.2), including column selection, the injection approach and temperature program, required optimisation in order to achieve thermostable analytes with sufficient chromatographic resolution, as well as to maximise injection volumes for analysis of low-level cellular sterols. A SIM method was then developed for the sensitive and selective quantification of each analyte (Section 4.2.5.3).

The first objective in the development of a COPs analysis method was to confirm if the GC-MS would offer satisfactory sensitivity to permit quantification using the method of Lee et al. (2008b). To this end, the original method was applied for the individual injection of each derivatised sterol standard at 1 µg, which additionally enabled confirmation of initial retention times and the corresponding mass spectra. The correct mass spectra and identity of any additional peaks were confirmed by a search against the National Institute of Standards and Technology (NIST) 70 eV EI mass spectral library.

### 4.2.5.1 Derivatisation

Optimisation of the derivatisation approach was required to improve analyte stability over time and remove decomposition peaks for 7α-HC, 7-KC and 25-HC (Section 4.3.1.1). Initial derivatisation of 5 µg of each difficult COP was performed in separate reactions using the original method (Section 4.2.5); stability of 7α-HC and 7-KC was evaluated over the course of ten repeat injections. Anhydrous sodium sulphate was used to remove residual moisture from pyridine and ethyl acetate prior to use (Section 4.2.4.3). To test if the presence of residual moisture was, regardless, interfering with silylation reactions, an azeotrope consisting of 40 µL toluene:methanol (1:1 v/v) was added to the dried standards and allowed to evaporate at 75 ºC in a dry block heater prior to derivatisation.

The derivatisation method was optimised to limit analyte decomposition based on reactions with only 7-KC as an indicative marker. A volume of 50 µL BSTFA (1 % v/v TMCS) was added to reactions, with or without an equal volume of pyridine, and analyte added at 5 or 1 µg to alter the reagent excess. After derivatisation, the reagent was evaporated with ultrapure nitrogen and the derivatised analyte reconstituted in ethyl acetate containing 10 or 40 % (v/v) reagent before injection. These reactions were evaluated by three key experiments that modified (1) incubation time and proportion of TMCS catalyst; (2) use of pyridine catalyst; and (3) reagent excess and proportion of reagent in the final injection volume, including direct injection
without reagent dilution (Table 4.2). A final analysis was performed based on the optimal procedure for limiting 7-KC decomposition, whereby a mixture containing 100 ng of each sterol (total mass below 1 µg) was derivatised with 200 µL BSTFA (1 % TMCS) with 1:1 (v/v) pyridine at 80 ºC for 2 hours, with reconstitution in 40 % (v/v) BSTFA (1 % TMCS) in ethyl acetate for injection.

Table 4.2: Derivatisation experiments (Exp.) with 7-ketocholesterol.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Reagent / reaction composition</th>
<th>Reagent excess</th>
<th>Injection solvent composition</th>
<th>Incubation temperature (ºC)</th>
<th>Incubation time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>BSTFA(^a) (20 % TMCS(^b))</td>
<td>10×</td>
<td>10 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>1B</td>
<td>BSTFA (1 % TMCS)</td>
<td>10×</td>
<td>10 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>1C</td>
<td>BSTFA (20 % TMCS)</td>
<td>10×</td>
<td>10 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>1D</td>
<td>BSTFA (1 % TMCS)</td>
<td>10×</td>
<td>10 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>2A</td>
<td>BSTFA (20 % TMCS):pyridine (1:1 v/v)</td>
<td>10×</td>
<td>10 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>2B</td>
<td>BSTFA (1 % TMCS):pyridine (1:1 v/v)</td>
<td>10×</td>
<td>10 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>2C</td>
<td>BSTFA (20 % TMCS):pyridine (1:1 v/v)</td>
<td>10×</td>
<td>10 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>2D</td>
<td>BSTFA (1 % TMCS):pyridine (1:1 v/v)</td>
<td>10×</td>
<td>10 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>3A</td>
<td>BSTFA (1 % TMCS)</td>
<td>50×</td>
<td>40 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>3B</td>
<td>BSTFA (1 % TMCS):pyridine (1:1 v/v)</td>
<td>50×</td>
<td>40 % (v/v) reagent in ethyl acetate</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>3C</td>
<td>BSTFA (1 % TMCS)</td>
<td>50×</td>
<td>Direct injection</td>
<td>80</td>
<td>120</td>
</tr>
</tbody>
</table>

\(^a\) N,O-bis(trimethylsilyl)trifluoroacetamide  
\(^b\) Trimethylchlorosilane
4.2.5.2 Instrument parameters

Preliminary evaluation of chromatogram peak resolution was achieved based on the method of Lee et al. (2008b), whereby 100 ng each derivatised sterol was injected onto a BPX5 (12 m × 0.22 mm × 0.25 µm) column with temperature programming in accordance with the original method (Section 4.2.5). In an attempt to improve resolution, this method was compared to injection of 50 ng each sterol onto a SLB-5ms (30 m × 0.25 mm × 0.25 µm) column, applying the same program with temperature ramping at 20 °C/min. A sequential modification of the temperature program using the latter column was made over the course of many injections in order to elute analytes with baseline separation of chromatographic peaks, resulting in the definitive method (Section 4.2.4.4). An initial column temperature of 80 °C enabled analytes to be ‘cold trapped’ at the start of the column to facilitate large volume injections, according to Section 4.2.4.4. Four replicate slow (1 µL/s), large volume (10 µL) injections of 10 ng of each analyte was compared to three replicate fast (50 µL/s), standard volume (2 µL) injections.

4.2.5.3 Selected ion monitoring

A SIM method was developed for the selective quantification of analytes. Calibration samples were prepared into blank or DMEM mock matrix samples (Section 4.2.3.1), each containing 100 ng of each analyte. The definitive methods were applied for sample preparation and GC-MS analysis (Section 4.2.4). Five replicate injections were made for 10 ng of each analyte on-column, which were detected by scanning over the entire m/z range (m/z = 60 to 1000) in order to identify abundant and characteristic ions. SIM elution windows for peak detection were selected, ideally, to include only a single peak; closely eluting peaks (within 1.5× the peak width at half the peak height) were grouped into a single window. A single ‘quantifier’ ion was selected for each analyte as the most abundant ion that was unique within its SIM window. Two additional abundant and characteristic ‘qualifier’ ions were also monitored, but were not necessarily unique between all peaks within a SIM window. The dwell time was adjusted based on the number of ions being detected within each window. The final SIM parameters are indicated in Section 4.2.4.4, Table 4.1.
4.2.6 Optimisation of a DNA and lipid co-extraction system

A modified solvent extraction system was designed based on the methods of Kehrmeyer et al. (1996) and Folch et al. (1957), applying a conventional lipid extraction system to co-extract DNA, while minimising the aqueous volume in order to enable DNA processing in 1.5 mL tubes. While these methods are common for the extraction of lipids, their reliability for simultaneous DNA extraction was unknown. The solvent extraction was unchanged from the definitive method (Section 4.2.4.1), except for the selection of aqueous buffer, which differed contingent on the stage of method development. After development of a cell lysis and extraction protocol, cell extracts were evaluated for DNA yield using aqueous buffer of various salinity (Section 4.2.6.1). The recovery of spiked DNA after solvent extraction and ethanol precipitation was then determined (Section 4.2.6.2). Finally, the co-extraction of endogenous sterols and DNA from PBMCs was evaluated (Section 4.2.6.3).

4.2.6.1 Cell lysis

Three cell lysis protocols were tested (Methods A, B and C), each starting with PBMC suspensions of 0.1 mL containing 1×10^6 cells. Cells underwent a single freeze-thaw (Method A) or two repeat freeze-thaws (Method B), with flash-freezing carried out in liquid nitrogen and thawing at room temperature. Cells were alternatively mixed with an equal volume of 3 M sodium chloride solution before being subjected to a single freeze-thaw (Method C). The treated cells were examined under a compound microscope (DM500, Leica Microsystems, Wetzlar, Germany) on a haemocytometer after staining with an equal volume of 0.4 % (w/v) trypan blue (Gibco by Life Technologies, Carlsbad, USA).

Cell lysis treatments were further evaluated by DNA yield after subjecting cells to solvent extraction (Section 4.2.4.1). Cell suspensions in 0.1 mL PBS were mixed with an equal volume of TE_1 buffer (Methods A and B) or high-salt TE_1 buffer (Method C). An untreated control containing equivalent volumes made up with PBS was prepared and extracted for comparison to cell treatments. Each procedure was repeated six times. The DNA yield after ethanol precipitation (Section 4.2.4.2.1) was measured with a Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit, according to the manufacturer’s protocol (Molecular Probes 2015).
4.2.6.2 Spiked DNA recoveries

An evaluation of DNA recovery after extraction was performed by spiking 480 ng G304A Human Genomic DNA (Promega, Madison, USA) into samples comprised of 0.1 mL PBS without the presence of cells. Extraction and ethanol precipitation of DNA was performed in accordance with the definitive methods (Sections 4.2.4.1 and 4.2.4.2.1). Aqueous dilution of sample extraction solvents to achieve phase separation was performed with either PBS (containing 1 mM EDTA) or TE$_1$ buffer to evaluate the impact of saline on overall DNA recovery from the aqueous phase. The optimal procedure was applied to samples carried through the entire DNA extraction process, with spiking prior to (1) extraction to find the entire method recovery; (2) ethanol precipitation to find the purification method recovery; and (3) Qubit analysis to verify detection accuracy in the extracted sample medium.

4.2.6.3 Preliminary co-extraction of endogenous cellular DNA and sterols

The optimal extraction system for DNA recovery (Section 4.2.4.1) was applied for the simultaneous extraction of endogenous DNA and selected sterols from PBMC suspensions in DMEM comprised of $5 \times 10^6$ cells. The DNA and sterol extracts were purified by ethanol precipitation (Section 4.2.4.2.1) and the original saponification/SPE (Section 4.2.7), respectively. Quantification of sterols (Section 4.2.4.5) was achieved from spiking of internal (19-HC) and recovery (betulin) standards at 10 ng each. DNA was quantified using the Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit, according to the manufacturer’s instructions (Molecular Probes 2015). DNA extracts from cell suspensions prepared in either PBS or DMEM were additionally applied to real-time PCR, in duplicate. Real-time PCR was performed using the Quantifiler Human DNA Quantification Kit (Applied Biosystems) with a 7500 Real-Time PCR System, following the manufacturer’s protocol (Applied Biosystems 2018).

4.2.7 Optimisation of saponification and SPE

A saponification and SPE protocol was adapted from the methods by Lee et al. (2008b), originally designed for direct saponification of plasma, for application to cellular lipid extracts. The original method included 1 mL plasma within the saponification mixture, as well as 2 mL each of the second wash (methanol/formic acid) and elution (hexane/ethyl acetate) solvents.
These parameters were modified during optimisations, substituting plasma with PBS as the basis for optimisation. Otherwise, the definitive method (Section 4.2.4.2.2) was unchanged from the original.

Sterol recoveries after saponification and SPE were used to evaluate the success of modified methods. Modifications to the SPE, performed in triplicate, included the application of 1.8 mL ammonium hydroxide, 1.8 mL methanol/formic acid or 2.5 mL hexane/ethyl acetate mixtures. The optimal combination of SPE solvent volumes (Section 4.2.4.2.2) was applied in duplicate to compare recoveries after saponification without aqueous dilution, as well as with dilution using either PBS or deionised water. Analyses were performed using blank samples spiked with 12 µg cholesterol and subjecting them to solvent extraction using the definitive method (Section 4.2.4.1). Sterols were spiked into extracted blank samples at 25 ng each with 5 ng internal/recovery standard. Additional samples, prepared without cholesterol, were extracted both with and without 50 µg BHT to verify any impact on sterol recoveries. Derivatised standards (Section 4.2.4.3) were reconstituted for injection of 2.5 ng of each analyte and 0.5 ng of internal and recovery standards onto the GC column. Quantification was performed with the definitive GC-MS method (Section 4.2.4.4 and 4.2.4.5).

4.2.8 Validation of the definitive extraction and GC-MS procedures

Evaluation of method sensitivity, quantitative limits, linearity, accuracy, precision, specificity and matrix effects was performed from the preparation of calibration curves (Section 4.2.8.1). Calibration samples comprised of blank or mock/DMEM matrix samples (Section 4.2.3.1), which were subjected to the entire sample preparation protocol prior to spiking of sterol standards at the stage of derivatisation, according to the definitive methods (Section 4.2.4). Samples were analysed using the definitive GC-MS/SIM method (Section 4.2.4.4). Corrections to the SIM parameters, based on changes to analyte retention times and SIM ion ratios between blank and mock matrix samples (Section 4.2.8.2), were required to ensure continued method specificity in the presence of DMEM matrix. Additionally, SIM mass spectra of calibration samples were compared to that of endogenous PBMC extracts. The definitive methods were then applied for determination of spiked sterol recoveries from both blank matrix and cell/DMEM matrix samples (Section 4.2.8.3).
4.2.8.1 Calibration curves

Calibration curves were prepared into blank or mock matrix sample extracts, which were extracted and analysed according to the definitive methods (Section 4.2.4). The blank matrix calibrations were evaluated as part of a preliminary examination in order to inform methodology for blank matrix recovery experiments (Section 4.2.8), which were performed several months in advance of mock matrix calibrations and recovery experiments.

The blank matrix calibration curves consisted of a ten-point standard dilution series spiked with 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1 ng of each analyte into silanised reaction vials for derivatisation. Internal standard (19-HC) and recovery standard (betulin) were spiked at a constant amount of 5 ng each. Derivatised standards were reconstituted in 100 µL for five replicate injections (10 µL) of 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01 ng of each analyte, and 0.5 ng internal and recovery standards, onto the GC column.

The mock matrix calibration curves comprised of a six-point dilution series prepared into duplicate mock matrix samples containing each sterol at 5, 2.5, 1, 0.5, 0.25 and 0.1 ng, with a constant amount of 10 ng internal and recovery standards. Derivatised standards were injected (10 µL) in duplicate from a 30 µL reconstitution volume for 1.67, 0.833, 0.333, 0.167, 0.083 and 0.033 ng of each analyte, and 3.33 ng of internal and recovery standards, on-column.

4.2.8.2 Method specificity

Calibration samples in blank and mock matrix, as applied for the generation of calibration curves (Section 4.2.8.1), were evaluated for retention time and mass spectral consistency. This was to assess and account for the effect of matrix on the detection specificity of each analyte. Retention time reproducibility was determined over all calibration sample dilutions and replicates. Mass spectral reproducibility, generated by SIM, was evaluated in triplicate for calibration samples diluted for the injection of ~0.03 ng of each analyte on-column. These were additionally compared to endogenous sterols extracted from PBMCs (cell matrix) spiked with 3.33 ng of internal (19-HC) and recovery (betulin) standard. The abundance of SIM ions relative to the base peak (100 % relative abundance) were compared between samples comprised of each matrix.
4.2.8.3 Spiked sterol recoveries

The definitive method (Section 4.2.4) for the extraction and analysis of selected sterols was evaluated for spiked sterol recoveries at each stage of the extraction method and at completion. This was determined by comparison of sterol quantities within pre-extraction versus post-extraction spiked samples. Samples included blank matrix spikes containing 12 µg cholesterol and PBMC suspensions in DMEM comprising 7.5×10^5 cells (Section 4.2.3). Analytes inclusive of 7α-HC, 7β-HC, 7-KC, 24-HC, 25-HC and 27-HC were spiked at 100 ng, while lathosterol was spiked at 1 µg, in order to inundate endogenous levels. Analytes were quantified from calibration curves corresponding to the sample matrix (Section 4.2.8.1). Derivatised standards were reconstituted in 100 µL for injection of 10 ng of each analyte (100 ng lathosterol), together with 0.5 ng (blank matrix samples) or 3.33 ng (DMEM/cell matrix samples) of internal/recovery standard onto the GC column. While the internal standard was added to samples at the same time as analytes, the recovery standard was added immediately prior to derivatisation, from which the recovery of the internal standard within each sample was determined.

Samples were prepared in triplicate with spiking prior to (1) extraction, to find the entire method recoveries; (2) saponification/SPE, to find the purification method recoveries; and (3) derivatisation, to verify detection accuracy in the extracted sample medium for each analyte. Additionally, (4) the sterol recovery from the extraction method alone was determined for blank samples by immediately following the solvent extraction of spiked analytes (without the presence of BHT or cholesterol) with derivatisation and GC-MS analysis.
4.3 Results

4.3.1 Optimisation of GC-MS analytical procedures

A preliminary analysis was conducted based upon an independent injection of each TMS-derivatised analyte, to determine initial retention times and characteristic mass spectral ions (Section 4.2.5). Seven out of ten analytes, including the internal and recovery standards, demonstrated a single peak corresponding to the compound of interest (Figure 4.1). The presence of multiple peaks in 7α-HC, 7-KC and 25-HC revealed the decomposition of these analytes prior to detection. These peaks were identified by comparison of mass spectra to the NIST 70 eV EI mass spectral library. The derivatisation procedure was optimised to completely derivatise these problem analytes into single peaks (Section 4.3.1.1). Modification of the temperature program and selected column was required to improve the separation of analytes, while the injection method was adjusted to concentrate trace-level analytes onto the GC column (Section 4.3.1.2). A SIM method was developed from the final GC-MS analysis procedure (Section 4.3.1.3).
Figure 4.1: Chromatograms and mass spectra for each analyte and standard. Derivatisation of each sterol was achieved in separate reactions with BSTFA (1% TMCS):pyridine (1:1 v/v) at room temperature for 2 hours. Sterols were injected at 1 µg for analysis by gas chromatography-mass spectrometry. Decomposition peaks identified by 70 eV mass spectral library search are also labelled.
Figure 4.1 (continued): Chromatograms and mass spectra for each analyte and standard. Derivatisation of each sterol was achieved in separate reactions with BSTFA (1% TMCS):pyridine (1:1 v/v) at room temperature for 2 hours. Sterols were injected at 1 µg for analysis by gas chromatography-mass spectrometry. Decomposition peaks identified by 70 eV mass spectral library search are also labelled.
4.3.1.1 Derivatisation

Analysis of each derivatised analyte separately by GC-MS (Section 4.2.5.1) revealed the formation of dehydration artefacts for 7α-HC, 7-KC and 25-HC; more dehydration occurred as derivatised products decomposed over time (Figure 4.2). The dehydration product comprised 39 % of the total signal from both the decomposition and silylation peaks for 7α-HC after a single injection and 93 % after ten injections. For 7-KC, the dehydration product accounted for 14 % of the total peak signals after a single injection and 37 % after ten injections. Decomposition of 25-HC with time was not monitored, but the dehydration product formed 42 % of the summed peak signal after a single injection. The use of an azeotrope to ensure the complete dryness of sterols prior to derivatisation did not improve these results (data not shown). Modification of the derivatisation method was made to improve analyte stability in order to minimise artefact formation and generate reproducible results over several injections.

**Figure 4.2: Decomposition of 7α-hydroxycholesterol (HC), 7-ketocholesterol (KC) and 25-HC upon gas chromatograph injection after derivatisation.** Derivatisation of 5 µg each sterol in separate reactions was achieved with BSTFA (1 % TMCS):pyridine (1:1 v/v) at room temperature for 2 hours. Decomposition of 7α-HC and 7-KC was evaluated over 10 repeat injections for analysis by gas chromatography-mass spectrometry.

Optimisation was performed using 7-KC and its dehydration product (3,5-cholestadien-7-one) as representative targets for derivatisation success (Figure 4.3). Three sets of experiments were performed testing various reaction compositions, reagent excess, reagent proportion in the injection solvent, and incubation time for derivatisation (Section 4.2.5.1, Table 4.2). The
summed peak signal for the dehydrated and silylated products, after BSTFA reactions with 1 % TMCS and 120 minute incubation at 80 ºC, revealed 60 % of the final product to be attributable to the TMS ether. The extent of successful derivatisation was worsened by the presence of 20 % TMCS and 30 minute incubations. An equivalent level of silylated product (61 %) was attained after 30 minutes in the presence of pyridine, which was slightly improved (66 %) after incubation for 120 minutes. Simultaneously increasing the reagent excess (from 10 to 40×) and the proportion of reagent in the final injection volume (from 10 to 40 %) increased the fraction of silylated product to 88 % without pyridine and 95 % with pyridine. Direct injection of derivatised product, without reagent evaporation and dilution upon reconstitution, did not improve the result (44 % 7-KC TMS ether).

![Figure 4.3: Proportion of dehydrated and silylated 7-ketocholesterol (7-KC) during optimisation experiments.](image)

Reactions were evaluated by three key experiments that modified (1) incubation time and proportion of trimethylchlorosilane (TMCS) catalyst (1A: 20 % TMCS for 30 minutes; 1B: 1 % TMCS for 30 minutes; 1C: 20 % TMCS for 120 minutes; 1D: 1 % TMCS for 120 minutes); (2) use of pyridine catalyst (under the same conditions as Experiment 1); and (3) reagent excess and proportion of reagent in the final injection volume (3A: with pyridine; 3B: without pyridine), including direct injection without reagent dilution (3C). Experiments are detailed in Section 4.2.5.1, Table 4.2.

Derivatisation of the entire mixture of sterols was performed with the optimal procedure designed to minimise 7-KC dehydration (Method 3B, Figure 4.3). Standards were added at lower amounts to achieve derivatisation reagent excess beyond 200 times, better representing the expected conditions for low concentrations of cellular sterols (Section 4.2.1). This final
derivatisation approach achieved complete derivatisation of all analytes to TMS ethers and was stable over the course of several injections (Figure 4.4).

Figure 4.4: Final derivatisation method stability for sterol mixtures. A mixture containing 100 ng each of 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 19-HC, 24-HC, 25-HC, 27-HC, cholesterol, lathosterol and betulin was derivatised with 200 µL BSTFA (1 % v/v TMCS) with 1:1 (v/v) pyridine at 80 ºC for 2 hours, with reconstitution in 40 % (v/v) BSTFA (1 % v/v TMCS) in ethyl acetate. Samples were evaluated over ten repeat injections for analysis by gas chromatography-mass spectrometry.

4.3.1.2 Instrument parameters

The GC temperature program was modified to improve the resolution of closely eluting peaks and to enhance the peak signal of late-eluting analytes (Section 4.2.5.2). Chromatograms were compared after using the original temperature program by Lee et al. (2008b) on a 12 m × 0.22 mm × 0.25 µm 5%-phenyl-PDMS column, a slower temperature ramp rate on a 30 m × 0.25 mm × 0.25 µm 5%-phenyl-PDMS column, and the final optimised method (Figure 4.5). The original method with a 12 metre capillary column exhibited a high baseline and did not sufficiently resolve 7-KC and 27-HC. This method, with an increased temperature ramp rate applied to a 30 metre column, did not sufficiently resolve lathosterol and 7β-HC, and generated a low peak signal for 7-KC. A final temperature program using the 30 metre column enabled baseline separation of all analytes and a more uniform peak signal for early- and late-eluting analytes.
Figure 4.5: Chromatograms generated after varying the temperature program and column length. A silylated mixture of 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 19-HC, 24-HC, 25-HC, 27-HC, cholesterol, lathosterol and betulin, was injected at 100 or 50 ng onto a 12m×0.22mm×0.25µm or 30m×0.25mm×0.25µm capillary column, respectively. Helium flow rate was maintained at 0.8 mL/min. Temperature programming was initially based on the method by Lee et al. (2008b): 160 ºC to 300 ºC at 40 ºC/min (12 m column) or 20 ºC/min (30 m column). The final, optimised method was 80 ºC (2 min) to 260 ºC (4 min) at maximum speed (initially 120 ºC/min), to 280 ºC (4 min) then 290 ºC (no hold) at 2 ºC/min, then to 300 ºC (1.5 min) at 4 ºC/min.

The final temperature method was simultaneously optimised for large volume injections by ‘cold trapping’ analytes for a period at 80 ºC at the start of the column. Large volume injections made slowly onto the GC column were assayed, along with standard mode, fast injections (Section 4.2.5.2). The chromatograms for repeated large volume injections were reproducible and consistent with that for standard mode injections (Figure 4.6).
Figure 4.6: Comparison of large volume injection and standard injection techniques. A silylated mixture of 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 19-HC, 24-HC, 25-HC, 27-HC, cholesterol, lathosterol and betulin, was injected at 10 ng slowly (1 µL/s) by large volume injections ($2 \times 5$ µL) or standard mode, fast injections (2 µL at 50 µL/s). Injections were repeated four times for large volume injections and three times for standard mode injections. (A) Peak area averages (± standard deviation) for each analyte; (B) overlaid chromatograms for large volume (black peaks) and standard (grey peaks) injections.

4.3.1.3 Selected ion monitoring

Once the analytes of interest were resolved chromatographically, a SIM method for quantification was established from injections of a blank-spiked calibration sample containing no matrix, as well as a calibration sample containing mock matrix composed of extracted cell suspension medium (Section 4.2.5.3). An improved sensitivity was attained by SIM, increasing the S/N for targeted analytes by more than 300 to 3000 times in comparison to scanning mode chromatograms (Figure 4.7). Chromatographic interferences were observed in the presence of DMEM matrix (Section 4.3.4.1), requiring the use of separate SIM retention windows for blank and matrix samples to account for retention time shifts (Section 4.3.4.2).
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4.3.2 Optimisation of a DNA and lipid co-extraction system

4.3.2.1 Cell lysis

An analysis of cell lysis procedures was conducted by freeze-thawing PBMC suspensions in PBS once (Method A) or twice (Method B), as well as by freeze-thawing after mixing with a high-salt solution (Method C) (Section 4.2.6.1). The extent of cell lysis was determined by comparison of cell counts to untreated cells (Figure 4.8). The number of observable cells was reduced by more than 90 % in all cases, with an almost complete loss of observable cells for Methods B and C.
Figure 4.8: Reduction of intact cells after lysis treatments relative to untreated cells. Cell lysis procedures for aqueous cell suspensions included use of a single freeze-thaw (Method A), two repeat freeze-thaws (Method B), and a single freeze-thaw with addition of 3 molar sodium chloride solution (Method C). Cell counts were conducted under compound microscope on a haemocytometer with trypan blue staining, and the extent of cell lysis determined from the absence of cells as compared to an examination of untreated cells. No repeat analyses were performed (n = 1).

Each of the cell lysis treatments were solvent extracted in the presence of either TE$_1$ buffer (Method A and B) or high-salt TE$_1$ buffer (Method C) (Figure 4.9). Following treatment, DNA yield was increased by the greatest extent using Method A and the least using Method C.

Figure 4.9: Increase in the yield of DNA after cell lysis treatments relative to untreated cells. Cell lysis procedures for aqueous cell suspensions included a single freeze-thaw with addition of a Tris-EDTA (TE) buffer (Method A), two repeat freeze-thaws with addition of TE buffer (Method B) and a single freeze-thaw with addition of a hypertonic buffer. Cells were extracted with a chloroform/methanol solvent system and DNA ethanol precipitated from the aqueous phase. The DNA yield was determined fluorometrically and expressed as a percentage increase relative to that of untreated cells. Six repeat analyses were performed (n = 6) and the mean ± standard deviation reported.
4.3.2.2 Spiked DNA recoveries

The recovery of spiked genomic DNA was measured following solvent extraction and ethanol precipitation of the aqueous phase (Section 4.2.6.2). The maximum attainable recovery (indicative of the spiking/quantification accuracy) was monitored by spiking DNA into extracted blank samples immediately prior to detection. Complete recovery was obtained for samples spiked just prior to detection, with about a 50 % DNA loss from the ethanol precipitation and another 10 % DNA loss from the total method (Figure 4.10).

![Figure 4.10: Recovery of spiked DNA from a lipid extraction system. Blank samples, comprised of phosphate-buffered saline, were prepared and carried through the solvent extraction system and ethanol precipitation of the aqueous phase. DNA was spiked at 480 ng into the pre-extraction medium (total method recovery), into the pre-purification medium (ethanol precipitation recovery) and into the pre-detection medium (maximum recovery). The mean recoveries ± standard deviation (n = 6) are reported.](image)

4.3.2.3 Preliminary co-extraction of endogenous cellular DNA and sterols

The final solvent extraction procedure, optimised for recovery of spiked DNA, was applied for the simultaneous extraction of endogenous DNA and various sterols from PBMC suspensions containing 5×10^6 cells (Section 4.2.6.3). The internal standard (19-HC) was recovered at 36.6 ± 8.48 % (mean ± standard deviation), with sterols and DNA detected at the amounts, and extrapolated for full recovery, as per Table 4.3. Poor peak shape / chromatographic interference prevented reliable peak integration for 7α-HC in all samples, as well as for a single replicate for 7β-HC, 7-KC and 25-HC. Both 24-HC and 27-HC could not be detected for a single replicate.
Table 4.3: Sterol and DNA quantity from co-extraction of peripheral blood mononuclear cell suspensions.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Replicate 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>19-HC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.41 (10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>2.74 (10)</td>
<td>3.82 (10)</td>
</tr>
<tr>
<td>7β-HC</td>
<td>Poor peak integration</td>
<td>0.17 (0.63)</td>
<td>0.18 (0.47)</td>
</tr>
<tr>
<td>7-KC</td>
<td>Poor peak integration</td>
<td>0.80 (2.92)</td>
<td>0.68 (1.79)</td>
</tr>
<tr>
<td>24-HC</td>
<td>0.03 (0.08)</td>
<td>Not detected</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td>25-HC</td>
<td>0.04 (0.09)</td>
<td>Poor peak integration</td>
<td>0.03 (0.08)</td>
</tr>
<tr>
<td>27-HC</td>
<td>0.09 (0.21)</td>
<td>Not detected</td>
<td>0.13 (0.34)</td>
</tr>
<tr>
<td>Lathosterol</td>
<td>2.81 (6.37)</td>
<td>2.77 (10.1)</td>
<td>2.83 (7.43)</td>
</tr>
<tr>
<td>DNA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.4</td>
<td>14.5</td>
<td>12.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Internal standard; recoveries were 44.1, 27.4 and 38.2 % (10 ng spikes)

<sup>b</sup> Extrapolated mass for 100 % recovery

Duplicate analysis of DNA by real-time PCR revealed yields of 5.64 and 8.21 ng/1×10<sup>6</sup> cells extracted from suspensions in PBS (data not shown). However, the DNA yield from cells suspended in DMEM could not be determined due to failed amplification. While the IPC assayed from cell extracts in PBS was consistently amplified with an approximate C<sub>q</sub> of 24.5, the IPC failed to amplify for cell extracts containing DMEM.

### 4.3.3 Optimisation of saponification and SPE

The saponification and SPE were optimised based on, and by comparison to, the procedure by Lee et al. (2008b) (Section 4.2.7). Modifications to the procedure were evaluated by spiked sterol recovery (Figure 4.11). Reducing the volume of the first SPE wash solvent (ammonium hydroxide) had little effect on the recovery of most sterols relative to the original procedure. This resulted in an average increase of 5.2 to 32 % for 7α-HC, 24-HC, 25-HC, 27-HC and lathosterol, but decreased average recoveries by 5.2, 14 and 60 % for 7β-HC, 7-KC and 19-HC (internal standard), respectively. A reduction of the second SPE wash solvent mixture (methanol and formic acid) increased the average recovery of the internal standard by 21 %, while raising average recoveries of all remaining sterols by 110 to 270 %. Increasing the volume of the final elution solvent mixture (hexane and ethyl acetate) raised the average recovery of the internal standard by 60 % and remaining sterols by 271 to 508 %. The presence of BHT during the SPE improved the average recoveries of each sterol by 82 to 513 %, with the average internal standard recovery being increased by 197 %.
Figure 4.11: Optimisation of the SPE for sterol recoveries. The original procedure by Lee et al. (2008b) applied 2 mL of each wash and elution solvent. The recoveries for each sterol were compared to the original method after application of 1.8 mL of each wash solvent, including ammonium hydroxide (Wash 1) and methanol/formic acid (Wash 2), and 2.5 mL of the final elution solvent mixture of hexane/ethyl acetate. The original method was performed with and without addition of 50 µg BHT. The original methods (with and without BHT) were analysed in triplicate (n = 3), while modifications to SPE solvent volumes were analysed in duplicate (n = 2). The mean recovery ± standard deviation for each sterol was measured by gas chromatography with mass spectrometric detection. The internal standard was 19-hydroxycholesterol (HC), with analytes including 7α-HC, 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol.

Dilution of the saponification solvent (methanolic potassium hydroxide) with aqueous plasma substitute reduced sterol recoveries (Figure 4.12). Recoveries with deionised water as diluent ranged 25.7 to 86.3 %, with a difference between duplicate measures of up to 29.9 %, and 48.5 to 73.2 % recovery of the internal standard. Recovery was also reduced by the use of PBS, ranging 29.7 to 61.8 %, with a difference between duplicate measures of less than 3 %, and 50.5 to 52.3 % recovery of the internal standard. Removal of any aqueous solution from the saponification allowed for recoveries of 43.1 % to 89.3 %, with a difference between duplicate measures of less than 10 %, and 69.4 to 75.0 % recovery of the internal standard.
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Figure 4.12: Effect of saponification mixture dilution on sterol recoveries. The original procedure by Lee et al. (2008b) was designed for direct saponification of plasma. Replacement of plasma was achieved with phosphate-buffered saline or deionised water and compared to undiluted saponification containing no plasma substitute. The recovery of each sterol was measured in duplicate (both results shown) by gas chromatography with mass spectrometric detection. The internal standard was 19-hydroxycholesterol (HC), with analytes including 7α-HC, 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol.

4.3.4 Validation of the definitive extraction and GC-MS procedures

4.3.4.1 Calibration curves

The quantitative limitations of the GC-MS were determined from the preparation of a dilution series containing a mixture of the sterol standards (Section 4.2.8.1). The resultant calibration curves were evaluated by their linear dynamic range (coefficient of determination, R²), accuracy (relative error of back-calculated quantity estimates) and precision (coefficient of variation). A LOD was evaluated as the lowest dilution capable of producing a detectable peak for all repeat injections, while a LOQ was the lowest dilution capable of producing a reproducible linear response for all repeat injections. The analytical limitations were evaluated for extracted blank matrix samples (Section 4.3.4.1.1) and DMEM matrix samples (Section 4.3.4.1.2). Matrix effects were apparent from chromatograms of similarly concentrated analytes (Figure 4.13).
Figure 4.13: Comparison of chromatograms with and without matrix effects. A silylated mixture of 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol was prepared in blank vials or mock matrix. Analyses were by selected ion monitoring. Internal (19-HC) and recovery (betulin) standards were injected at 0.5 ng from blank samples and 3.33 ng from matrix samples. (A) Blank sample containing 1 ng of each analyte; (B) blank sample containing 0.025 ng of each analyte; (C) matrix sample containing 1.67 ng of each analyte; (D) matrix sample containing 0.033 ng of each analyte.

4.3.4.1.1 Blank (no matrix) calibrations

Calibration curves were generated for each target sterol, without sample matrix interferences, by spiking into blank vials for immediate derivatisation (Section 4.2.8.1). Reliable quantification (LOQ) was established from a 10 ng on-column injection, with linearity (R² ≥ 0.993) down to 0.25 ng for 7-KC, 0.05 ng for lathosterol, and 0.025 ng for 7α-HC, 7β-HC, 24-HC, 25-HC and
27-HC (Figure 4.14). Most sterols were detectable (LOD) for all repeat injections down to the lowermost dilution of 0.01 ng on-column, except for 24-HC and 27-HC, which were undetectable for three out of five injections at the lowermost dilution, imparting an LOD of 0.025 ng for these sterols.

**Figure 4.14: Calibration curves without matrix effects.** Sterols were serially diluted prior to derivatisation for five repeat injections \((n = 5)\) of 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01 ng of each analyte, including 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol. Internal standard (19-HC) was added at a constant amount for injection of 0.5 ng. A linear relationship was found for the logarithm of the weight ratio of the analyte to internal standard \((W_x/W_y)\) plotted against the logarithm of the peak area ratio of the analyte to internal standard \((A_x/A_y)\). The limit of detection (LOD) and limit of quantification (LOQ) were determined from the calibration curves. The linear equation and coefficient of determination \((R^2)\) are displayed for the quantitative range (dark grey points), excluding detectable, but non-linear dilutions (light grey points).
The accuracy and precision of calibration curves for estimating quantities over the linear dynamic range was determined from back-calculations relative to the expected, spiked mass (Figure 4.15). The relative error of quantity estimates was below 10 or 20% for most analyte dilutions, with errors (mean ± 95% confidence interval) over the entire linear range of 10.5 ± 7.4% for 7α-HC, 7.9 ± 2.6% for 7β-HC, 9.0 ± 5.5% for 7-KC, 8.6 ± 3.6% for 24-HC, 10.0 ± 7.5% for 25-HC, 8.9 ± 6.5% for 27-HC and 6.6 ± 4.2% for lathosterol. The overall coefficient of variation was 7.2 ± 1.5% for 7α-HC, 2.9 ± 1.7% for 7β-HC, 5.3 ± 2.6% for 7-KC, 6.0 ± 2.9% for 24-HC, 11.4 ± 3.4% for 25-HC, 7.9 ± 3.8% for 27-HC and 4.0 ± 1.8% for lathosterol. The mass estimate of the internal standard (19-HC), injected at constant amount throughout all analyte dilutions, was determined from injection of a recovery standard (betulin) and its relative response factor (Section 4.2.4.5). The relative error of 19-HC was consistent with analytes, with an overall error of 9.3 ± 3.9% and coefficient of variation of 6.4 ± 1.5%.

![Figure 4.15: Accuracy and precision of the sterol quantification from calibration curves without matrix effects.](image)

Sterols were serially diluted prior to derivatisation for five repeat injections (n = 5) of 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01 ng of each analyte, including 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol. The internal standard (19-HC) was injected at constant amount (0.5 mg) in all analyte dilutions and its recovery derived from response factors for a recovery standard (betulin). The mean ± standard deviation of the estimated analyte mass was derived from calibration curve back-calculations, expressed as a percentage relative to the expected spiked mass (indicated by the solid line).
4.3.4.1.2 Mock matrix calibrations

Calibration curves were generated for each sterol to include matrix effects by spiking into the extract of freeze-dried cell suspension medium (DMEM) prior to derivatisation (Section 4.2.8.1). Calibration curves were linear ($R^2 \geq 0.985$) from injections of 1.67 ng on-column down to a LOQ of 0.167 ng for 7-KC, 25-HC and lathosterol, 0.083 ng for 7α-HC and 7β-HC, and 0.033 ng for 24-HC (Figure 4.16). The linearity of 27-HC was only achieved at 0.956 ($R^2$) down to an injection of 0.083 ng onto the GC column. Except for 7-KC, which was undetectable for one out of four injections, all sterols were detectable (LOD) to the lowermost dilution of 0.033 ng.
Figure 4.16: Calibration curves with matrix effects. Sterols were serially diluted prior to derivatisation for duplicate injections from duplicate extracted matrix samples (n = 4) of 1.67, 0.833, 0.333, 0.167, 0.083 and 0.033 ng of each analyte, including 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol. Internal standard (19-HC) was added at a constant amount for injection of 3.33 ng. A linear relationship was found for the logarithm of the weight ratio of the analyte to internal standard (\( W_x/W_y \)) plotted against the logarithm of the peak area ratio of the analyte to internal standard (\( A_x/A_y \)). The limit of detection (LOD) and limit of quantification (LOQ) were determined from the calibration curves. The linear equation and coefficient of determination (\( R^2 \)) are displayed for the quantitative range (dark grey points), excluding detectable, but non-linear dilutions (light grey points).
The accuracy and precision of quantity estimates from calibration curves over the linear dynamic range was determined from back-calculations relative to the expected, spiked mass (Figure 4.17). The relative error of quantity estimates was less than 10 or 20 % for most analyte dilutions, with errors (mean ± 95 % confidence interval) over the entire linear range of 9.1 ± 8.1 % for 7α-HC, 4.8 ± 2.5 % for 7β-HC, 6.3 ± 5.2 % for 7-KC, 6.1 ± 4.4 % for 24-HC, 5.4 ± 7.1 % for 25-HC, 18.0 ± 9.0 % for 27-HC and 4.6 ± 4.8 % for lathosterol. The overall coefficient of variation was 7.7 ± 7.1 % for 7α-HC, 5.1 ± 3.3 % for 7β-HC, 6.3 ± 6.5 % for 7-KC, 9.2 ± 5.3 % for 24-HC, 6.8 ± 4.6 % for 25-HC, 9.0 ± 3.7 % for 27-HC and 3.6 ± 4.1 % for lathosterol. The mass estimate of the internal standard (19-HC), injected at constant amount throughout all analyte dilutions, was determined from injection of a recovery standard (betulin) and its relative response factor (Section 4.2.4.5). The relative error of 19-HC was consistent with analytes, with an overall error of 5.3 ± 3.3 % and coefficient of variation of 7.2 ± 4.1 %.

**Figure 4.17: Accuracy and precision of the sterol quantification from calibration curves with matrix effects.** Sterols were serially diluted prior to derivatisation for duplicate injections from duplicate extracted matrix samples (n = 4) of 1.67, 0.833, 0.333, 0.167, 0.083 and 0.033 ng of each analyte, including 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol. The internal standard (19-HC) was injected at constant amount (3.33 ng) in all analyte dilutions and its recovery derived from response factors for a recovery standard (betulin). The mean ± standard deviation of the estimated sterol mass was derived from calibration curve back-calculations, expressed as a percentage relative to the expected spiked mass (indicated by the solid line).
4.3.4.2 Method specificity

To account for matrix effects on the detection specificity of each analyte, chromatograms and mass spectra of calibration samples in each matrix were compared (Section 4.2.8.2). A retention time shift was observed in the presence of DMEM matrix, slowing analyte elution by 40 to 70 seconds. For calibration samples containing the same matrix, retention times were reproducible, with coefficients of variation below 0.3 % for all analytes (Table 4.4).

Table 4.4: Retention time reproducibility of blank and mock matrix samples.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Blank samples (n = 48)</th>
<th>Matrix samples (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean retention time (minutes)</td>
<td>Coefficient of variation (%)</td>
</tr>
<tr>
<td>7α-HC</td>
<td>18.26</td>
<td>0.11</td>
</tr>
<tr>
<td>19-HCa</td>
<td>20.51</td>
<td>0.10</td>
</tr>
<tr>
<td>Lathosterol</td>
<td>21.16</td>
<td>0.09</td>
</tr>
<tr>
<td>7β-HC</td>
<td>25.17</td>
<td>0.12</td>
</tr>
<tr>
<td>24-HC</td>
<td>25.79</td>
<td>0.12</td>
</tr>
<tr>
<td>25-HC</td>
<td>26.25</td>
<td>0.19</td>
</tr>
<tr>
<td>7-KC</td>
<td>27.38</td>
<td>0.15</td>
</tr>
<tr>
<td>27-HC</td>
<td>29.24</td>
<td>0.10</td>
</tr>
<tr>
<td>Betulinb</td>
<td>18.26</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*internal standard; b recovery standard

Mass spectra generated by SIM also demonstrated matrix effects by comparison of blank and mock (DMEM) matrix-spiked samples, as well as endogenous (non-spiked) sterols in extracted cell matrix. Comparisons were made for spiked analytes of approximately equal concentration, toward the low-end of calibration curves (~0.03 ng on-column). Changes to the relative abundance of SIM ions were observed for analytes in extracted DMEM and cell matrix samples, which were greater than the variation observed for that of blank samples (Figure 4.18). In comparison to blank samples, a resultant decrease (average ± standard deviation) in relative abundance was consistently attained for DMEM matrix samples with and without cells for 19-HC (m/z = 145), lathosterol (m/z = 255), 24-HC (m/z = 413 and 129), and 7-KC (m/z = 129). An increase in relative abundance was evident for DMEM matrix samples with and without cells for 7β-HC (m/z = 441), 27-HC (m/z = 417 and 456), and betulin (m/z = 496). The ion ratios relevant to each sample matrix were applied for quantitative SIM analyses in order to retain specificity for each analyte.
Figure 4.18: Relative abundance of mass spectral ions in different matrices. A silylated mixture containing 7α-hydroxycholesterol (HC), 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol was injected at ~0.03 ng of each analyte from blank vials or mock matrix, in triplicate (n =3). A triplicate injection of endogenous sterols from extracted cell matrix was also performed. Internal (19-HC) and recovery (betulin) standards were injected at 0.5 ng from blank matrix samples and 3.33 ng from mock matrix samples. Three mass spectral ions for each analyte were monitored by selected ion monitoring, and method specificity evaluated by changes to the abundance of each ion relative to the ‘base peak’ ion (100 % relative abundance; indicated under each analyte name) in the presence of each matrix. The average relative abundance ± 95 % confidence interval is reported.

4.3.4.3 Spiked sterol recoveries

The final optimised procedure for the extraction and purification of various sterols, from both blank matrix and PBMC suspensions, was applied for the determination of spiked sterol recoveries (Section 4.2.8.3). Recoveries were measured for various stages of the analytical procedure (Figure 4.19). The average recovery (± standard deviation) from the solvent extraction of blank-spiked samples ranged from 64 ± 1.4 to 92 ± 1.2 % across all sterols, with 91 ± 2.4 % recovery of the internal standard. Recovery from the solvent extraction alone could not be monitored for PBMC-spiked samples due to a requirement for sample cleanup prior to detection. The recoveries for blank-spiked samples after saponification and SPE were 36 ± 4.8 to 82 ± 7.0 % for analytes and 64 ± 5.6 % for the internal standard. These recoveries ranged from 32 ± 2.2 to 84 ± 5.1 % for analytes spiked into PBMC suspensions, with 66 ± 3.6 %
recovery of the internal standard. The total method recoveries ranged from 29 ± 2.6 to 68 ± 1.9 % across analytes spiked into blank samples and 37 ± 7.8 to 82 ± 12 % for analytes spiked into PBMC suspensions. The internal standard was recovered from the entire analytical process at 56 ± 5.0 % for blank samples and 66 ± 6.8 % for PBMC samples.

Figure 4.19: Recoveries of spiked sterols from the final extraction and purification approach. Spiked sterols were extracted from blank samples containing no cellular material, and PBMC suspensions containing 7.5×10^5 cells. The mean recovery ± standard deviation for each sterol was measured in triplicate (n = 3) by gas chromatography with mass spectrometric detection, following solvent extraction, saponification/SPE and the total method. The internal standard was 19-hydroxycholesterol (HC), with analytes including 7α-HC, 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol.

The accuracy of recovery measurements based on the detector response was monitored by spiking sterols into the extracted sample medium from blank and PBMC samples immediately prior to derivatisation. The recoveries from these samples were determined in the same manner as all recovery experiments and compared to the theoretical recovery of 100 % (Figure 4.20). Analyte recoveries ranged from 83 ± 4.2 to 113 ± 2.2 % for blank-spiked samples and 92 ± 1.9 to 123 ± 1.5 % for PBMC-spiked samples. Recoveries of the internal standard were 86 ± 0.1 % and 88 ± 2.8 % for blank and PBMC samples, respectively.
Figure 4.20: Accuracy of the spiked sterol recoveries. Sterols were spiked subsequent to extraction immediately prior to derivatisation into blank samples containing no cellular material, and PBMC suspensions containing 7.5×10^5 cells. The accuracy was determined from the back-calculated recovery of each sterol, in comparison to the theoretical recovery of 100% (indicated by the solid line). The mean recovery ± standard deviation for each sterol was measured in triplicate (n = 3) by gas chromatography with mass spectrometric detection. The internal standard was 19-hydroxycholesterol (HC), with analytes including 7α-HC, 7β-HC, 7-ketocholesterol (KC), 24-HC, 25-HC, 27-HC and lathosterol.

4.4 Discussion

4.4.1 Optimisation and validation of quantitative GC-MS analytical procedures

Large volume injection techniques are a prerequisite for concentrating trace components onto the GC column (Hoh & Mastovska 2008). A large volume injection procedure was developed for the GC-MS analysis of expected trace-level COPs (Section 4.2.1), as well as the cholesterol precursor, lathosterol, together with 19-HC (internal standard) and betulin (recovery standard). Near identical consistency was obtained as compared to standard volume injections (Section 4.3.1.2, Figure 4.6). The temperature program was also modified from that of Lee et al. (2008b) for application to a 30 metre capillary column with improved resolution as compared to the original temperature program and 12 metre column, which did not completely resolve the
analytes of interest under the conditions/instrumentation applied in this study (Section 4.3.1.2, Figure 4.5).

Following derivatisation of these selected sterols to TMS ethers for GC-MS analysis, three problematic analytes were identified that produced an additional dehydration product at greater abundance than the silylated product (Section 4.3.1.1, Figure 4.1 and Figure 4.2). This included 7α-HC (dehydrated to 4,6-cholestadien-3-ol), 7-KC (dehydrated to 3,5-cholestadien-7-one) and 25-HC (dehydrated to 5,24-cholestadien-3-ol). These dehydration products, particularly for 7-KC, are indicative of thermal decomposition, which may occur upon GC injection due to incomplete derivatisation (Busch & King 2009). Steric hindrance of the 25-hydroxyl group increases the difficulty of its derivatisation, although the use of BSTFA (1 % v/v TMCS) at 60 °C for 1 hour or at room temperature for 24 hours was previously able to completely silylate 25-HC to its bis-TMS ether (Lai et al. 1995). This could also be attained after 30 minutes at room temperature by inclusion of the base catalyst, pyridine (Guardiola et al. 2004). Lee et al. (2008b) successfully derivatised these sterols with the same BSTFA/TMCS mixture at room temperature for 120 minutes in the presence of pyridine.

Various derivatisation methods were evaluated over the course of the GC-MS method workup to minimise or prevent hydroxyl dehydration, including modification to incubation time and temperature, derivatisation reagent excess, use of pyridine catalyst, and the proportion of derivatisation reagent within the final injection solvent (Section 4.3.1.1, Figure 4.3). Of these variables, increasing reagent excess during incubation and in the final injection solvent had the greatest effect on derivatisation success. While a 1:1 molar ratio of TMS donor to active hydrogen is the stoichiometric requirement for hydroxyl silylation and a 2× reagent excess is recommended (Supelco 1997), a 10× reagent excess of BSTFA (1 % TMCS) was not sufficient for complete derivatisation in this study. Although recommended for difficult analytes (Supelco 1997), this was not improved by the use of 20 % TMCS, likely due to dilution of the principal reactant. Near complete derivatisation was only attainable after incubation of sterol mixtures at 80 °C in the presence of 40× BSTFA (1 % TMCS) excess, together with pyridine, for 120 minutes; complete derivatisation was achieved by applying 200× reagent excess (Section 4.3.1.1, Figure 4.4). Non-concordance of this result with previous studies may suggest the use of a weak reagent batch (Busch et al. 2011); however, the same result was attained using reagent acquired from various batches and manufacturers (data not shown). It is therefore unknown why complete derivatisation was only possible under such extreme conditions,
considering the silylation reagents, 5%-phenyl-PDMS column, GC separation and MS detection techniques that were employed are common to COPs analysis (Sections 1.4.3 and 1.4.4).

The final derivatisation and GC-MS analysis protocols were used to create a SIM method, which increased sensitivity by 300 to 3000 times across all analytes (Section 4.3.1.3, Figure 4.7). The presence of DMEM matrix, in comparison to blank matrix, was shown to interfere with chromatograms, resulting in additional peaks (Section 4.3.4, Figure 4.13) as well as retention time shifts (Section 4.3.1.3, Table 4.4); however, retention times were reproducible within the same matrix. The ratios of mass spectral ions generated by SIM were also influenced, consistently, by the presence of DMEM extracted both with and without cells (Section 4.3.4.2, Figure 4.18). This indicated that the DMEM extract, rather than cells, was the predominant cause of mass spectral interference. The SIM method was adjusted to account for the retention time and mass spectral changes that were introduced by the DMEM matrix, preserving specificity for each analyte in each matrix to ensure correct peak designation.

The linearity of calibration curves (Section 4.3.4.1) was generally reduced in the presence of DMEM matrix ($R^2$ from 0.985 to 0.996; Figure 4.16), as compared to samples derivatised without extracted matrix ($R^2$ from 0.993 to 0.998; Figure 4.14). Only 27-HC spiked into extracted DMEM produced a poorly linear result ($R^2 = 0.956$). Despite the blank-spiked samples being diluted beyond the range of the matrix-spiked samples, a loss in sensitivity/linearity was apparent in the presence of DMEM matrix. This is due to the LOD/LOQ for blank-spiked samples being attainable at the lowermost dilutions that were applied to such samples (0.01 or 0.025 ng on-column), while the matrix-spiked LOD/LOQ were often above the lowermost dilution that was applied (0.033 ng on-column). Only lathosterol exhibited a blank-spike LOQ (0.05 ng on-column) within range of the matrix-spiked dilution series (0.167 ng on-column), which supported the sensitivity reduction observed in DMEM.

While peak area and sterol concentration, each relative to that for the internal standard, were proportional without DMEM (i.e. without matrix effects), this was not the case with DMEM (i.e. matrix effects included). This is evident when the near 1:1 relative proportionality of signal to concentration for all sterols in blank matrix (Figure 4.14) is compared with the non-linear proportionalities (slopes < 1) in DMEM matrix (Figure 4.16). Thus, with DMEM in the matrix, an increase in sterol concentration was not accompanied by the same relative increase in peak area. This could be expected to result in reduced quantification accuracies for sterols in DMEM; organic components of DMEM were therefore considered to have been carried through the SPE
that interfered with COP detection signals (discussed in Section 6.4). However, the linear relationship between relative peak area and concentration was preserved when data were log transformed.

Quantitative accuracy was slightly lower for blank-spiked samples within the linear range (Figure 4.15) than that for DMEM matrix-spiked samples (Figure 4.17). For instance, the relative error of the internal standard was reduced from 9.3 ± 3.9 % for blank-spiked samples to 5.3 ± 3.3 % for matrix-spiked samples. This trend was not followed for 27-HC, due to its poor linearity, which demonstrated potential for relative errors greater than 20 %. Conversely, precision was generally reduced in the presence of DMEM matrix, attributing to a marginally increased coefficient of variation for the internal standard from 6.4 ± 1.5 % for blank-spiked samples to 7.2 ± 4.1 % for matrix-spiked samples. However, the overall accuracy and precision demonstrated relative errors and coefficient of variation within 10 to 20 % over most analyte dilutions irrespective of matrix type.

### 4.4.2 Optimisation of a DNA and lipid co-extraction system

Freeze-thawing and use of high-salt (3 M sodium chloride) solution was evaluated for cell lysis of PBMCs (Section 4.3.2.1, Figure 4.8). Freeze-thawing is known to increase cell permeability and cause lysis from the formation of ice crystals within the cell (Kar & Singhal 2015), while hypertonic conditions (> 0.15 M) can result in lysis by osmotic shock and cell permeation by salt influx (Zade-Oppen 1968). Freeze-thawing cells twice in isotonic buffer (PBS) and once with the application of hypertonic solution resulted in similar levels of cell lysis, reducing the number of observable cells by about 99 % or more relative to cell counts of untreated cells. Use of a single freeze-thaw in PBS also resulted in about a 90 % reduction in observable cells in comparison to an untreated control. However, these results are in contrast to observations following a solvent extraction procedure to evaluate cell lysis on the basis of DNA yield.

The greatest average increase in DNA yield, as compared to untreated cells, was observed after a single freeze-thaw of cells suspended in a 1:1 (v/v) mixture of PBS (isotonic) and TE (hypotonic) buffer (~ 10-fold increase), with the lowest increase ascribed to hypertonic samples (~ 2.6-fold increase) (Section 4.3.2.1, Figure 4.9). It is possible that this change to DNA yield is not only attributable to cell lysis, but also due to differences in extraction efficiency that is dependent on salt concentration. For instance, the presence of high salt concentrations may precipitate DNA out of the aqueous phase in accordance with the same principles of ethanol
precipitation (Chen et al. 2010; Tan & Yiap 2009). A second freeze-thaw with TE buffer increased DNA yield from untreated controls by about half that of a single freeze-thaw (~ 5.6-fold increase). A reduced DNA quantity after a second rapid freeze-thaw is consistent with DNA losses observed after sample freezing (Ross et al. 1990). A single freeze-thaw, minimising addition of excess salt, was selected as being the optimal procedure for cell lysis.

Applying the optimal extraction buffers, the recovery of DNA from blank-spiked samples (Section 4.3.2.2, Figure 4.10) demonstrated the greatest loss after ethanol precipitation, at about 50%. The solvent extraction itself only accounted for an additional ~10% loss in DNA recovery, thereby recovering spiked DNA at about 90% from the solvent extraction alone. The detection method did not impact recoveries, with post-extraction spiked samples recovering near 100% of the spiked DNA. Thus, the lipid extraction system was successfully adapted for DNA recovery from spiked samples when matrix effects were absent. Such effects were evaluated by applying this procedure in a preliminary co-extraction of endogenous DNA and selected sterols from cellular material.

The lipid extraction procedure optimised for DNA recovery demonstrated a capacity for the simultaneous isolation of both sterols and DNA from PBMCs (Section 4.3.2.3, Table 4.3). Chromatographic issues concerning peak shape and interference in the presence of cellular material prevented peak integration for several analytes in some replicates. This likely resulted from the use of a non-optimal saponification and SPE approach at this stage of the method development. For sterol peaks that could be properly integrated, the total extrapolated quantities were often lower than expected, except for 7-KC, which was consistent with its expected mass, and 7β-HC and 27-HC, which were present at greater amount than anticipated. This may reflect different relative proportions of these COPs in PBMCs versus erythrocytes, which formed the basis of quantity estimations (Section 4.2.1). However, the primary problem with the co-extraction approach was the limited recovery of cellular DNA and its amenability for PCR in the presence of DMEM extract.

In the presence of PBMCs, which could be expected to contain ~7 picograms (pg) of DNA per cell (Gillooly et al. 2015), DNA was poorly recovered using this method. The total DNA yield, as measured by Qubit fluorometry, ranged from 12.6 to 30.4 ng per 1×10⁶ cells. The yield of amplifiable nuDNA, as measured by real-time PCR, was only 5.64 to 8.21 ng per 1×10⁶ cells when cells were suspended in PBS prior to extraction. Extraction of cells suspended in DMEM resulted in PCR inhibition, suggesting the carryover of aqueous DMEM components, which is
detrimental to the PCR. This inhibitory effect was likely exacerbated by the concentration of water-soluble components from 5 mL freeze-dried DMEM into a small aqueous volume (0.1 mL) for extraction. An enhanced purification approach is likely necessary to eliminate these effects (discussed in Section 6.4).

4.4.3 Optimisation of saponification and SPE

Prior to optimisation, spiked sterols were recovered from the SPE at approximately 10 % or below for all analytes. Optimisation proceeded by reducing the volume of wash solvents to minimise analyte loss, as well as by increasing the volume of the final elution solvent to ensure complete recovery of analytes from the SPE cartridge (Section 4.3.3, Figure 4.11). Reduction in the volume of ammonium hydroxide solution applied to the SPE sorbent demonstrated little improvement to recoveries, or otherwise worsened the recovery. This wash step may therefore be critical to remove interferents that impede sterol retention during further sorbent washes. A reduction in the volume of the methanol/formic acid mixture raised sterol recoveries by more than two-fold, suggesting this wash step to be a primary contributor to inadvertent analyte loss, likely due to increased hydrophobicity introduced by the methanol. Similarly, application of a larger volume of hexane/ethyl acetate elution mixture resulted in a greater than three-fold increase to sterol recoveries, demonstrating increased contact of the elution solvent and sorbent to improve the final elution efficiency. The presence of BHT also raised sterol recoveries, lending to a three-fold increase in the recovery of internal standard. Under the conditions applied during SPE, BHT (pK_a > 10) will remain neutral and thus could be retained by the lipophilic sorbent until elution by the methanolic wash (Krasowska et al. 2001). By this action, BHT may compete for lipophilic retention with interferents that would otherwise impede COP retention, or act to preferentially dissolve into the methanolic wash ahead of COPs, maximising COP retention to the sorbent.

Recoveries were further improved by removing any aqueous medium from the saponification (Section 4.3.3, Figure 4.12). The original saponification approach was designed for direct application to 1 mL plasma samples, which resulted in aqueous dilution of the saponification mixture. However, substituting plasma with aqueous solution was detrimental to sterol recoveries. This was likely due to reduced solubility of sterols into the methanolic saponification solvent in the presence of aqueous diluent. This would not occur for plasma
samples, considering endogenous hydrophobic sterols will be dissolved into plasma by association with lipoproteins (Feingold & Grunfeld 2015).

4.4.4 Spiked sterol recoveries from the definitive method

Spiking of each analyte into blank matrix samples revealed almost complete recovery from the solvent extraction procedure alone, with about 90% recovery of the internal standard (Section 4.3.4.3, Figure 4.19). The saponification/SPE contributed to most analyte losses, with 64 ± 6.0% average recovery (± standard deviation) of the internal standard. Recoveries were also evaluated by spiking analytes into PBMC suspensions at amounts of at least 100× the expected endogenous concentrations. This was to prevent any significant endogenous contribution to recoveries. Similar recoveries were attained for spiked PBMCs in comparison to blank-spiked samples, with 66 ± 3.6% recovery of internal standard from the saponification/SPE. Recoveries from the total method were around 10% greater for each sterol spiked into PBMCs than blank samples. It is possible that the presence of cell matrix improves sterol recoveries from the solvent extraction by inclusion of components (e.g. salts) that increase organic partitioning of lipid species (Breil et al.). The detection response of analytes in the sample medium (Section 4.3.4.3, Figure 4.20) was accurate to within 10 to 20%, consistent with those previously determined (Section 4.3.4).

4.5 Summary and conclusions

A large volume injection technique was developed for the GC-MS separation and analysis of selected COPs, including 7α-HC, 7β-HC, 7-KC, 24-HC, 25-HC and 27-HC, as well as the cholesterol precursor, lathosterol. The internal standard for analyte quantification was 19-HC, with betulin as recovery standard. Thermal decomposition of 7α-HC, 7-KC and 25-HC was problematic for quantitative analysis, but was resolved by derivatisation with at least a 200× reagent excess in the presence of pyridine base catalyst and by maintaining 40% (v/v) reagent in the final injection solvent. Calibration curves were prepared for quantitative SIM analysis without cell matrix and in a simulated matrix containing freeze-dried DMEM. Matrix effects were observed in the presence of DMEM, including shifts to retention times and mass spectral ion ratios, which were corrected for in the final analyses. A loss of sensitivity and linearity was apparent when analytes were spiked into DMEM extract, although similar accuracies and
precision were attained between blank- and matrix-spiked samples (within 10 to 20 % relative error and coefficient of variation). However, 27-HC was poorly linear in DMEM, with potential for inaccuracies beyond a 20 % relative error.

Co-extraction of both DNA and these selected sterols was possible with about 90 % recovery of each from the solvent extraction alone. The purification process to isolate DNA and sterol fractions resulted in the greatest recovery losses, with about 40-50 % loss of DNA and sterols after ethanol precipitation and SPE, respectively. While the sterol recoveries were improved in the presence of cell matrix, attributing to around 10 % higher recoveries, endogenous DNA was recovered vastly below expected yields at 13 to 30 ng per 1×10^6 cells. Although adequate for PCR application, real-time PCR was inhibited in the presence of DNA ‘purified’ from DMEM extract. Time constraints prevented further optimisation of a DNA cleanup procedure to remove inhibitors. Similarly, the developed sterol extraction and analysis methods suffered limitations in the presence of DMEM matrix that could not be completely resolved and validated due to time constraints, although were considered to be sufficient for quantification of COPs after low-dose gamma-irradiation (Chapter 5).
Chapter 5: Formation of cholesterol oxidation biomarkers in human cells after gamma-irradiation
5.1 Introduction

Exposure of an individual to ionising radiation may result following a radiological incident that may arise accidentally or criminally. These individuals may include victims or bystanders, the first responders to the scene, as well as the perpetrators of a radiological crime. The radiation dose received by an individual can inform the severity of acute radiation syndromes (progressing from 0.5 to beyond 15 Gy, Section 1.3.3), as well as the potential for stochastic outcomes, such as cancer incidence (Garau et al. 2011; Zeegers et al. 2017). Thus, radiation injury triage appreciably benefits from the determination of an individual’s biological exposure dose, which may furthermore act to associate persons of interest with a radiological crime (Section 1.3.3.1).

A predominant pathway for ionising radiation interaction with cellular constituents, particularly for radiations that embody a low LET type (e.g. gamma-radiation), involves the induction of ROS as mediators of cellular oxidative damage (Sections 1.3 and 1.3.1). This can occur through water radiolysis, as well as radiation-induced upregulation of mitochondrial oxidative function (Leach et al. 2001; Yamamori et al. 2012). The cell adaptive response to such stress, or radiation hormesis (Section 1.3.3), includes induction of antioxidants, DNA repair pathways and cell death or senescence to prevent replication of mutagenic effects (Azzam et al. 2012; Feinendegen et al. 2004; Tubiana et al. 2009). The extent of the cell adaptation proceeds in a dose-dependent manner between 1 to 500 mGy (Section 1.3.3).

Biomolecular damage and the altered expression of compounds involved in the cell stress response are therefore useful biological markers of ionising radiation exposure (Section 1.3.3.1). Current biomarkers that have demonstrated a capacity to serve as biodosimetric indicators include proteins (e.g. cytokines, γ-H2AX), peripheral blood cell counts, chromosomal aberrations, RNAs, and metabolites (Guipaud & Benderitter 2009; Singh et al. 2016; Zeegers et al. 2017). Oxysterols (or COPs) are produced through the addition of an oxygen group (e.g. hydroxyl, ketone, epoxide) to the steroid ring or aliphatic tail (side-chain) of the cholesterol structure (Gill et al. 2008; Kulig et al. 2016; Olkkonen et al. 2017; Valenzuela et al. 2003). These compounds may function in the cellular radiation response and thus also serve as biodosimetric markers.

Metabolic cholesterol oxidation (Section 1.3.2.2.1, Figure 1.9) is carried out by cholesterol hydroxylases, most of which belong to the microsomal and mitochondrial cytochrome P450 superfamily (Nebert & Russell 2002). Enzymatic oxidation is typically localised to the aliphatic
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tail, generating compounds such as 24-HC, 25-HC and 27-HC (Gill et al. 2008; Kulig et al. 2016; Olkkonen et al. 2017; Valenzuela et al. 2003). Additionally, the unsaturated bond between carbons 5 and 6 is susceptible to autoxidation in the presence of ROS to form cholesterol 5α-hydroperoxide, which stabilises via decomposition to 7α-HC or 7β-HC, or dehydration to 7-KC (Gill et al. 2008; Murphy & Johnson 2008; Terao 2014; Valenzuela et al. 2003). However, certain COPs, such as 7α-HC and 25-HC, can originate from both oxidation pathways (Gill et al. 2008). Oxygen addition can also arise at many other sites along the cholesterol side-chain, forming COPs such as 20-HC, 22-HC, and 24,25-epoxycholesterol (EC), as well as within the steroid rings, forming 4β-HC, 5,6-EC, and 3β,5α,6β-CT, as examples (Gill et al. 2008; Kulig et al. 2016; Murphy & Johnson 2008).

COPs are associated with an abundance of physiological roles, for example, as metabolic intermediaries, as well as in gene expression and cell signalling for processes including cell survival and cell death (Kulig et al. 2016; Vurusaner et al. 2016). Many of these functions are exerted through the agonistic action of several COPs, including 7α-HC, 24-HC, 25-HC and 27-HC, on LXRs, a family of nuclear receptors that modulate gene expression (Gamba et al. 2015). The plasma levels of some of these COPs, including 7α-HC, 24-HC and 27-HC, have been demonstrated, together with other novel and conventional markers, to enable estimation of tissue damage and absorbed dose in cases of accidental exposure (Bertho et al. 2009; Bertho et al. 2008). The production of such COPs may also be modified by changes to the expression of the 18 kilodalton (kDa) translocator protein (TSPO), which is considered to mediate cholesterol import into the mitochondria (Papadopoulos et al. 2006; Papadopoulos et al. 2017; Papadopoulos & Miller 2012). Ligands of TSPO are also thought to be associated with radiation-induced effects, such as apoptosis and ROS formation (Betlazar et al. 2016; Gatliff et al. 2014; Lin et al. 2014; Liu et al. 2017; Veenman & Gavish 2012).

This study monitored the production of various COPs, and the cholesterol precursor, lathosterol, in human cells after exposure to doses of gamma-radiation ranging from 1 to 10,000 mGy. The irradiated cells were wild type Jurkat cells with low to absent TSPO expression, TSPO overexpressed Jurkat cells (Liu et al. 2017), as well as PBMCs. Extracted, purified and silylated COPs/sterols were measured by GC-MS to evaluate biomarkers of gamma-radiation exposure, as well as any dose correlation and/or biological significance.
5.2 Methodology

5.2.1 Chemicals and standards

Sterol standards and chemicals were purchased in accordance with Section 4.2.2.

5.2.2 Cell cultivation and sample preparation

5.2.2.1 Jurkat cells

Human T lymphoma Jurkat cell lines, including wild type (low/absent TSPO), TSPO overexpressed (TSPO+) and empty plasmid transfection control cells were prepared at ANSTO, with plasmid construction and cell transfection performed as described by Liu et al. (2017). Cells were cultured and maintained in high glucose DMEM (D6429, Sigma-Aldrich), supplemented with 10 % (v/v) foetal bovine serum (Invitrogen) and 2 mM glutamine (Sigma-Aldrich), in 5 % carbon dioxide at 37 °C. Stable transfections were maintained with the addition of 500 mg/mL Geneticin (Thermo Fisher Scientific, Waltham, USA). Cells were counted using a Countess Automated Cell Counter (Invitrogen) with 0.4 % (w/v) trypan blue. Triplicate 5 mL aliquots of approximately 1×10^6 cells/mL were prepared in silanised Pyrex 9 mL culture tubes with phenolic screw caps for each radiation dose. Cultured cells were aliquoted for irradiations on the same day.

5.2.2.1.1 Peripheral blood mononuclear cells

Whole blood (~80 mL) was collected from each of three individuals (all male, ~25 years of age) by venepuncture into multiple lithium heparin-coated 10 mL Vacutainers (Becton Dickinson). Cells were prepared from whole blood on the same day as collection. Whole blood collected from a single healthy donor was pooled into sterile 50 mL tubes and diluted 1:1 (v/v) with PBS. A density gradient centrifugation was carried out to isolate the PBMCs using Ficoll-Paque Plus, performed in accordance with Section 4.2.3.2. A 5 µL aliquot of resuspended cells was diluted 50× with PBS and cells were counted on a haemocytometer after staining with an equal volume of 0.4 % (w/v) trypan blue to confirm cell viability. Sample aliquots (approximately 1×10^6 cells/mL in 5 mL DMEM) were prepared in triplicate for each radiation dose into silanised
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Pyrex 9 mL culture tubes. Cells were prepared the day prior to irradiations, stored in 5 % carbon dioxide at 37 °C overnight, then transported the following day from the University of Canberra to ANSTO at ambient temperature (~20 to 25 °C).

5.2.3 Sample irradiation

Gamma-irradiation of cell suspensions was conducted at ANSTO. Irradiation to absorbed doses of 1, 10 and 100 mGy were performed using a Theratron $^{60}$Co low-dose irradiator (Atomic Energy of Canada Limited, Chalk River, Canada), while doses of 5000 and 10,000 mGy were performed using a $^{60}$Co high-dose irradiator (GATRI). Irradiations were carried out independently over a continuous period for each dose at ambient temperature (~17 °C). Allowance was made for the decay of the radioactive source in calculating exposure times.

Jurkat cells underwent a preliminary set of irradiations to approximate absorbed doses of 10, 100 and 5000 mGy. Sample tubes for the Theratron irradiator were positioned on a slab of Solid Water (Gammex, Middleton, USA) to achieve the required dose rate, as well as at a slight incline to prevent the solution from contacting the plastic cap. The dose rate was approximately 34.2 mGy/min with an approximate 2 % variation across samples. This dose rate was determined from a previous dose mapping experiment (data not shown) with a calibrated ionisation chamber inserted into a machined cavity in the Solid Water. Sample tubes for the high-dose irradiator were placed in an esky filled with polyethylene beads at a position previously determined from a dose mapping study to deliver a dose rate approximating 1.5 Gy/min. Specifically, exposure times were 18 seconds for 10 mGy, 175 seconds for 100 mGy, and 3.4 minutes for 5000 mGy.

PBMCs were irradiated to the complete dose range, from 1 to 10,000 mGy. Irradiations were performed in the same manner as for Jurkat cells, with the 10 and 100 mGy samples irradiated at approximately 32.1 mGy/min, the 1 mGy samples irradiated at 15.0 mGy/min, and the higher doses irradiated at 1.38 Gy/min. Therefore, exposure times varied for each dose from 4 seconds (1 mGy) up to around 7 minutes (10,000 mGy).

All samples were flash frozen in liquid nitrogen immediately after irradiation and stored at -80 °C. Transport to the University of Canberra was on dry ice.
5.2.4 Sample extraction and analysis

Extraction and analysis of targeted sterols was performed in accordance with the definitive developed methods (Section 4.2.4). Frozen samples were freeze-dried prior to solvent extraction in the presence of BHT (Section 4.2.4.1). Saponification of the organic fraction and SPE were then performed to isolate the COPs/sterols of interest (Section 4.2.4.2.2). The sterols were derivatised to TMS ethers (Section 4.2.4.3) for quantitative analysis by GC-MS (Sections 4.2.4.4 and 4.2.4.5).

5.2.5 Statistical analysis

Variation in the recovered mass between each triplicate set of unirradiated controls (Jurkat cells), or up to four unirradiated controls per individual (PBMCs), was evaluated as the percentage divergence (from 100 % concordance) from the most concentrated replicate in the series. To account for any variation between unirradiated controls, the irradiated samples were compared against each appropriate control separately, and the data pooled for statistical analysis using SPSS Statistics 23. A 5 % significance level was applied to all tests and asymptotic (two-tailed) \( p \)-values obtained, where a \( p \)-value of less than 0.05 was considered a statistically significant result. Divergence from a normal Gaussian distribution was checked using Shapiro-Wilk normality tests and confirmed by Q-Q plots. Equality of variances were checked using Levene’s test. Extreme values were identified and removed from the data analysis if they were beyond the first or third quartile of the dataset by more than \( 1.5 \times \) the interquartile range.

Nonparametric tests for related samples (repeated measures) were applied for evaluation of any dose-effects within data grouped by cell type (Jurkat or PBMC) and cell line/donor. These analyses were conducted using Friedman’s tests, with multiple comparisons made by Wilcoxon Signed-Rank tests. Mann-Whitney U tests were used to compare the effect of cell line/donor at equivalent doses, as well as the dose-effect between analytes. Sequential Bonferroni (Holm-Bonferroni) corrected significance levels were employed to adjust \( p \)-values in order to mitigate against chance significance due to multiple comparisons. In cases that were significant prior to Holm-Bonferroni correction, but not after, both corrected and uncorrected \( p \)-values were reported.
5.3 Results

5.3.1 Variation of unirradiated controls

The precision of each replicate unirradiated control (triplicate for Jurkat cells and up to four replicates for PBMCs) was measured as the mass ratio, pooled across all analytes, relative to the most concentrated replicate in the series (Figure 5.1). Unirradiated Jurkat cells were $72.7 \pm 23.5\%$ (mean ± standard deviation) concordant in their recovered sterol mass for wild type cells, $61.5 \pm 31.0\%$ for TSPO+ cells, and $88.9 \pm 12.6\%$ for empty plasmid cells. Unirradiated PBMCs were approximately $42-60 \pm 20-26\%$ concordant in their recovered sterol mass, dependent on donor, which accounted for a pooled variation of $54.6 \pm 38.5\%$ for all individuals. To account for this variation in the irradiated sample analysis, the relative change of irradiated samples, which were determined separately against each control, was pooled.

![Figure 5.1: Precision of replicate sterol measurements in unirradiated cells.](image) Cells included Jurkat cell lines (wild type, translocator protein overexpressed (TSPO+), and empty plasmid control cells), and peripheral blood mononuclear cells (PBMCs) collected from three individuals (Person A, B and C). Variation in the measured mass for all sterols between preparations of triplicate Jurkat cells and up to four replicate PBMCs was determined as the mass ratio of each replicate to a single replicate in the series, represented as a percentage, where 100 % is a fully concordant (precise) result. Displayed are the average (± standard deviation) results for all analytes ($n = 12$ for all Jurkat cells; 6 for Person A and B; 3 for Person C; and 15 for pooled PBMCs).
5.3.2 Cholesterol oxidation in Jurkat cells

Jurkat cell lines, including wild type, TSPO+ and empty plasmid transfection control cells, were exposed to low doses of gamma-radiation (10, 100 and 5000 mGy). The quantities of various COPs and cholesterol precursor, lathosterol, were measured in snap-frozen irradiated cells for comparison against that of unirradiated control cells (Figure 5.2). Recovery of 27-HC was below the LOQ (< 8.33 pg/µL, Section 4.3.4.1.2) in all cases and therefore not examined. The concentrations of most analytes isolated from control cells were similarly distributed between cell lines; however, the concentration range (represented by the mean ± standard deviation) demonstrated an elevated trend in unirradiated TSPO+ cells in comparison to unirradiated wild type cells for both 24-HC (wild type: 1.37 ± 0.25 ng/1×10⁶ cells; TSPO+: 2.49 ± 0.78 ng/1×10⁶ cells) and lathosterol (wild type: 83.9 ± 13.8 ng/1×10⁶ cells; TSPO+: 384 ± 74.8 ng/1×10⁶ cells). The concentration range of wild type cells typically overlapped between samples irradiated to different doses, except for 24-HC after 10 mGy (2.81 ± 0.97 ng/1×10⁶ cells) and lathosterol after both 10 mGy (455 ± 177 ng/1×10⁶ cells) and 100 mGy (165 ± 42.9 ng/1×10⁶ cells). This was attributed to a significant and consistent increase in the total mass, relative to control cells (mean ± 95 % confidence interval), after 10 mGy for both 24-HC (by 110 ± 54.1 %, \( p = 0.023 \)) and lathosterol (by 451 ± 154 %, \( p = 0.023 \)), as well as after 100 mGy for lathosterol (by 99.7 ± 40.2 %, \( p = 0.023 \)). The overall concentration of 24-HC and lathosterol in wild type cells progressively decreased, relative to unirradiated controls, as dose increased, which was significant for lathosterol at 5000 mGy (by 18.9 ± 12.6 %, \( p = 0.023 \)).
Figure 5.2: Measurement of selected oxysterols and precursor sterol (lathosterol) in normal and gamma-irradiated Jurkat cell lines. Jurkat cells, including wild type, translocator protein overexpressed (TSPO+), and empty plasmid transfection controls, were exposed to doses of 10, 100 or 5000 milligray (mGy) of gamma-radiation, in triplicate. Figure A shows the mass in nanograms (ng) of sterol derivatives (trimethylsilyl) per 1 million cells for each replicate, as measured by gas chromatography-mass spectrometry. Separate y-axis scales are applied for sterols of different concentration ranges. Figure B shows the pooled relative changes in analyte mass of irradiated cells from each unirradiated control (indicated by the solid line at 0 %), comprising between six and nine observations at each dose/analyte (6 ≤ n ≤ 9). Extreme values are 1.5× the interquartile range beyond the first or third quartile. Asterisks denote statistically significant differences (Wilcoxon-Signed Rank, p < 0.05) after Sequential Bonferroni correction (**), as well as significant differences prior to correction, but not after (*).
Unlike wild type cells, the empty plasmid cells demonstrated a significant reduction, in comparison to unirradiated controls, in the production of all sterols after irradiation at 10 mGy (7α-HC, by 37.0 ± 6.15 %, \(p = 0.023\); 24-HC, by 27.1 ± 4.32 %, \(p = 0.023\); 25-HC, by 30.0 ± 13.4 %, \(p = 0.023\); lathosterol, by 40.1 ± 6.5 %, \(p = 0.023\)) and/or 100 mGy (7α-HC, by 49.1 ± 11.8 %, \(p = 0.023\); by 7β-HC, 24.7 ± 12.3 %, \(p = 0.033\); by 7-KC, 50.2 ± 14.8 %, \(p = 0.023\); by 25-HC, 32.2 ± 19.5 %, \(p = 0.042\)). An exception was lathosterol after 100 mGy, which increased in recovered mass (180 ± 44.5 %, \(p = 0.023\)). A substantial, but nonsignificant change was attained at 10 mGy for 7-KC of wild type (189 ± 136 % increase, \(p = 0.114\) or 0.038 before correction) and empty plasmid cells (34.6 ± 27.8 % decrease, \(p = 0.076\) or 0.038 before correction). TSPO+ cells raised the production of most sterols from that of empty plasmid cells back to within the level of variation for wild type cells, resulting in no significant effects as compared to unirradiated controls. The exception was 24-HC and lathosterol, which significantly decreased in concentration from that of controls at 10 mGy (24-HC, by 37.4 ± 14.9 %, \(p = 0.023\); lathosterol, by 73.2 ± 3.52 %, \(p = 0.023\)) and 100 mGy (24-HC, by 46.7 ± 17.4 %, \(p = 0.023\); lathosterol, by 82.2 ± 3.80 %, \(p = 0.023\)).

To examine the effect of TSPO overexpression without any influence of plasmid transfection, the response (as measured by the relative change of irradiated cells from unirradiated controls) of the empty plasmid cells was subtracted from that of the TSPO+ cells. An overall significant increase in response (mean ± 95 % confidence interval) relative to the unirradiated controls was observed for some sterols (Figure 5.3A). This occurred after 10 mGy for 7α-HC (by 52.3 ± 30.8 %, \(p = 0.035\)), with a nonsignificant trend toward an increase observed at 100 mGy (by 38.4 ± 26.7 %, \(p = 0.071\) or 0.036 before correction). This same trend was apparent for 25-HC at 10 mGy (by 56.9 ± 59.5 %, \(p = 0.083\) or 0.038 before correction) and 100 mGy (by 87.6 ± 102.4 %, \(p = 0.083\) or 0.028 before correction). Conversely to wild type cells, overall significant decreases were present for lathosterol after 10 mGy (by 33.2 ± 4.8 %, \(p = 0.023\)) and 100 mGy (by 262 ± 41.8 % decrease, \(p = 0.023\)) and 24-HC at 100 mGy (72.1 ± 15.8 % decrease, \(p = 0.023\)). Subsequently, the overall response was significantly lowered for plasmid-subtracted TSPO+ cells, as compared to wild type cells (Figure 5.3B), after 10 mGy for both 24-HC (by 54.1 ± 13.9 %, \(p < 0.000\)) and lathosterol (by 86.7 ± 3.22 %, \(p < 0.000\)) and 100 mGy for both 24-HC (by 74.5 ± 14.9 %, \(p < 0.000\)) and lathosterol (182 ± 15.4 %, \(p < 0.000\)). However, overall 24-HC concentration was significantly increased in plasmid-subtracted TSPO+ cells, relative to wild type cells, after 5000 mGy (by 56.1 ± 45.0 %, \(p = 0.016\)), as was 25-HC after 100 mGy (by 42.6 ± 31.2 %, \(p = 0.006\)).
Figure 5.3: Comparison of cholesterol oxidation product and precursor sterol (lathosterol) recovery in wild type and plasmid-subtracted translocator protein overexpressed (TSPO+) Jurkat cell lines after gamma-irradiation. Jurkat cells, including wild type, TSPO+, and empty plasmid transfection controls, were exposed to doses of 10, 100 or 5000 milligray (mGy) of gamma-radiation, in triplicate. The response of empty plasmid cells, as measured by the relative change of irradiated cells from unirradiated controls, was subtracted from that of TSPO+ cells. Figure A shows the pooled change in recovered mass relative to that of unirradiated controls (indicated by the solid line at 0%) for wild type and plasmid-subtracted TSPO+ cells, comprising between six and nine observations at each dose/analyte (6 ≤ n ≤ 9). Figure B shows the change in the response of plasmid-subtracted cells relative to that of wild type cells (indicated by the solid line at 0%). Extreme values are 1.5× the interquartile range beyond the first or third quartile. Asterisks denote statistically significant differences from unirradiated controls (Wilcoxon-Signed Rank, p < 0.05; Figure A) and between cell type (Mann-Whitney U, p < 0.05; Figure B) after Sequential Bonferroni correction (**), as well as significant differences prior to correction, but not after (*).
The frequencies of analytes that increased or decreased in concentration (relative to the unirradiated controls) and the extent of such change, was consistent with the aforementioned significant results (Figure 5.4). For wild type cells, an increase from background levels was apparent for all observations after 10 mGy for both 24-HC (56 % ≥ 100 % increase) and lathosterol (100 % ≥ 100 % increase), and after 100 mGy for lathosterol only (44 % ≥ 100 % increase). Quantities for plasmid-subtracted TSPO+ cells were consistently increased from unirradiated controls for 7α-HC at 10 mGy (11 % ≥ 100 % increase) and 25-HC at 100 mGy (33 % ≥ 100 % increase), while all samples were reduced in relative mass for 24-HC after 100 mGy (78 % ≥ 50 % decrease), and for lathosterol after both 10 mGy (0 % ≥ 50 % decrease) and 100 mGy (100 % ≥ 50 % decrease). While these changes were not consistent for most analytes, an increase in concentration after irradiation was most frequent, including increases greater than two-fold the concentration of unirradiated cells. This was most prevalent after a 10 mGy dose to wild type cells, which increased the relative mass of each of the C7-oxidised COPs in 77.8 % of cases (66.7 % of 7-KC ≥ 100 % increase), as well as the tail-oxidised COPs, 25-HC in 66.7 % of cases and 24-HC in every case. However, remaining cases were reduced in concentration, lending to inconsistent results.

![Figure 5.4: Frequency and extent of samples that increased or decreased in recovery of cholesterol oxidation products or precursor sterol (lathosterol) after gamma-irradiation relative to unirradiated controls. Jurkat cells, including wild type, translocator protein overexpressed (TSPO+), and empty plasmid transfection controls, were exposed to doses of 10, 100 or 5000 milligray (mGy) gamma-radiation, in triplicate. The response of empty plasmid cells, as measured by the relative change of irradiated cells from unirradiated controls, was subtracted from that of TSPO+ cells (a solid line is shown at 50 %).](image)
5.3.3 Cholesterol oxidation in PBMCs

PBMCs isolated from the blood of three individuals were gamma-irradiated to doses ranging from 1 to 10,000 mGy. The production of various COPs and cholesterol precursor, lathosterol, was measured in the snap-frozen isolates (Figure 5.5A). A high level of variation in the concentrations of each analyte, for each individual, attributed to overlap in the concentration range between different doses. Measured quantities were below the LOQ (Section 4.3.4.1.2) in many cases across dose (including unirradiated controls) for each analyte; 15 % of cases for 7α-HC (< 16.7 pg/µL), 6 % for 7β-HC (< 8.33 pg/µL), 12 % for 7-KC (< 16.7 pg/µL), 21 % for 24-HC (< 3.33 pg/µL), 50 % for 25-HC (< 16.7 pg/µL), 86 % for 27-HC (< 8.33 pg/µL), and 3 % for lathosterol (< 16.7 pg/µL). The response to irradiation, as measured by the change in sterol mass relative to the unirradiated controls, was often upwards of a 100 % increase after irradiation to any dose (Figure 5.5B); increases beyond 1000 % were observed in single cases for Person 2 after 10 mGy (24-HC, 25-HC, 7-KC, lathosterol) and 100 mGy (7-KC, lathosterol), and for Person 3 after 5000 mGy in a single case for 7-KC, which were removed as outliers (data not shown). However, the measured concentration was not consistent between individuals, nor within the same individual, with these same doses and analytes also yielding reductions in total mass from that of unirradiated controls.
Figure 5.5: Measurement of selected oxysterols and precursor sterol (lathosterol) in normal and gamma-irradiated human peripheral blood mononuclear cells. Cells were collected from three individuals and exposed to doses of 1, 10, 100, 5000 or 10,000 milligray (mGy) of gamma-radiation. Figure A shows the mass in nanograms (ng) of sterol derivatives (trimethylsilyl) per 1 million cells for each individual, as measured by gas chromatography-mass spectrometry. Separate y-axis scales are applied for sterols of different concentration ranges. Figure B shows the pooled relative changes in analyte mass of irradiated cells from each unirradiated control (indicated by the solid line at 0 %), comprising between one and four observations at each dose/analyte (1 ≤ n ≤ 4). Extreme values are 1.5× the interquartile range beyond the first or third quartile.
To account for interindividual variation, the relative changes determined for each individual were pooled (Figure 5.6A). Concentrations of both 25-HC and 27-HC below the LOQ in most samples excluded them from further analysis. For all measured analytes, no significant differences in recovery were observed after irradiation to any dose, due to a large variation in the dose-response. This resulted in cases that increased in mass relative to controls, often by more than 100%, as well as cases that decreased, sometimes by more than 50% (Figure 5.6B).

**Figure 5.6:** Pooled recovery of cholesterol oxidation products and precursor sterol (lathosterol) in human peripheral blood mononuclear cells after gamma-irradiation. Cells were collected from three individuals and exposed to doses of 1, 10, 100, 5000 or 10,000 milligray (mGy) of gamma-radiation. Figure A shows the pooled change across individuals in the analyte mass of irradiated cells relative to that of unirradiated controls (indicated by the solid line at 0%), comprising between four and nine observations at each dose/analyte (4 ≤ n ≤ 9). Extreme values are 1.5× the interquartile range beyond the first or third quartile and are labelled by individual (1, 2 or 3). Figure B shows the frequency and extent of sample increases and decreases in the recovery of each sterol relative to unirradiated controls (a solid line is shown at 50%).
While the pooled change in analyte mass was highly variable, the mass of ring-oxidised COPs increased (mean ± 95 % confidence interval) by the greatest extent, typically proceeding from 1 mGy (7α-HC, 37.0 ± 60.5 %; 7β-HC, 72.2 ± 78.9 % and 7-KC, 81.1 ± 113 %) to 100 mGy (7α-HC, 101 ± 92.1 %; 7β-HC, 205 ± 181 % and 7-KC, 218 ± 275 %), with doses beyond 100 mGy having a lesser effect. For these analytes, observations of post-irradiation sterol mass increases, relative to controls, peaked after 100 mGy for 7α-HC (86 % of cases with 57 % ≥ 100 % increase), 7β-HC (89 % of cases with 67 % ≥ 100 % increase), and 7-KC (86 % of cases with 57 % ≥ 100 % increase). The concentration range of the tail-oxidised COP, 24-HC did not vary substantially between each dose; however, irradiation did typically result in a reduction in the mass of 24-HC from unirradiated controls. The exception was 10 mGy, which exhibited a relative increase in 56 % of samples (33 % ≥ 100 % increase). Cholesterol precursor, lathosterol, exhibited the greatest and most widely distributed changes at 10 mGy (82.8 ± 106.9 % increase), increasing from controls in 67 % of cases (42 % ≥ 100 % increase). Otherwise, the mass of 24-HC and lathosterol produced a highly varied response with the tendency to decline with dose overall, yielding an approximately equal number of increases as decreases in comparison to controls (slightly skewed toward decreases). For most analytes, only around 10-20 % of samples changed in mass from unirradiated controls by more than a 50 % reduction after irradiation. However, no significant changes in the measured quantities between analytes irradiated to an equivalent dose were observed.

5.4 Discussion

The pathophysiological functions of COPs, inclusive of apoptosis, cytotoxicity, cell differentiation and inflammation, have been implicated in conditions such as atherosclerosis, neurodegenerative diseases, inflammatory bowel diseases, diabetes and several cancers (Olkkonen et al. 2012; Poli et al. 2013; Sabuncuoğlu & Öztas 2014; Vejux et al. 2008). The pathogenesis of these conditions involves oxidative cellular stress, characterised by ROS imbalance, and thus COPs have been implicated to underlie the aetiologies of such disease states. Formation of ROS is central to ionising radiation-induced biological damage (Azzam et al. 2012; Leach et al. 2001; Yamamori et al. 2012), such that may occur during radiotherapy or in the event of exposure due to a radiological incident of an accidental or criminal nature. Gamma-radiation poses a high-risk for harmful exposures due to its high penetrative power, not requiring ingestion or inhalation to cause biological injury, and common use of gamma-
emitters, such as $^{137}$Cs, $^{192}$Ir and $^{60}$Co, in medical and industrial applications (Ferguson et al. 2005). COPs are composite indicators of both metabolic and ROS-mediated changes occurring within the cell (Noguchi et al. 2014; Nury et al. 2013), and their production after irradiation may infer cellular radiation responses and act as biodosimetric markers.

The production of COPs in human cells after gamma-irradiation was evaluated using the GC-MS methodologies devised in Chapter 4. Of the COPs examined, the ring-oxidised COPs, $7\alpha$-HC, $7\beta$-HC, and 7-KC, produced predominantly via autoxidation in the presence of cellular ROS, are recognised to reduce cell viability in neuronal cells due to cytotoxic activity (Nury et al. 2013). The tail-oxidised (aliphatic side-chain) COPs, 24-HC, 25-HC and 27-HC, derived primarily through enzymatic action, have instead been correlated with the induction of adaptive protection in neuronal and Jurkat cells in response to oxidative cell stress, including activation of apoptotic/necroptotic cell death (Gamba et al. 2015; Noguchi et al. 2014; Okabe et al. 2014). The biosynthesis of cholesterol necessary for the formation of such COPs can be monitored through the production of lathosterol (Duane 1995; Olkkonen et al. 2017).

The influence of TSPO on COP production was also examined in stable transfected Jurkat cell lines. TSPO is an outer mitochondrial membrane protein that has been incorporated, together with the steroidogenic acute regulatory protein (StAR), into the prevailing model for cholesterol transport from the cytosol into the mitochondria for steroid hormone synthesis (Papadopoulos et al. 2006; Papadopoulos & Miller 2012). This model has been contested by later evidence (Banati et al. 2014; Morohaku et al. 2014; Tu et al. 2014), suggesting TSPO is non-essential for cholesterol transport and as a vital protein for life as was the previous connotation (Papadopoulos et al. 1997). Consequently, the exact molecular mechanisms and functions of TSPO in many of its processes is of current debate (Selvaraj & Stocco 2015; Selvaraj et al. 2015; Šileikytė et al. 2014). However, it has been theorised that the observed controversies, specifically involving normal functionality of TSPO knock-out mice, are due to the replacement of TSPO with redundant mechanisms that permit continued animal viability (Papadopoulos et al. 2017). In addition to cholesterol transport for mitochondrial steroid/sterol synthesis, TSPO has been implicated in apoptosis and ROS production (Gatliff et al. 2014; Veenman & Gavish 2012). Therefore, increased TSPO expression was hypothesised to facilitate cytotoxic biological effects in response to gamma-radiation. In particular, it was thought that mitochondrial ROS production and cholesterol import would be increased to result in the heightened cellular generation of COPs.
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A large degree of variation was observed between sterol measurements within unirradiated control cells, which appeared greater for PBMCs and TSPO+ cells than wild type and empty plasmid Jurkat cells (Figure 5.1). This variation may be due to matrix interference introduced by the presence of cell suspension medium (DMEM; Section 4.3.4), or due to measurement errors associated with an imperfect GC-MS system (discussed in Section 6.4 and 6.5). However, the accuracy and precision of calibration samples (Section 4.3.4.1) and spiked sterol recoveries (Section 4.3.4.3) were within or below 10 to 20 % across all analytes for blank and matrix samples. The variation of sterols between unirradiated cell replicates may therefore indicate matrix effects due to the presence of cells that influence sterol measurements at trace levels. Such effects would not have been accounted for by validation experiments with mock matrix (cell-free) or cell matrix (spiked with sterol concentrations beyond endogenous levels). Appropriate experiments are required to more accurately determine the method limitations due to the sample matrix, which were not performed due to time and budgetary constraints (Section 6.5). To account for this variation in the unirradiated controls, the relative change of irradiated samples was determined separately against each control and then pooled for analysis of irradiation results. While pooled results were highly variable, some consistencies allowed for significant findings.

5.4.1 Sterol production in Jurkat cells

The production of sterols was compared in Jurkat cell lines, including wild type (low/absent TSPO), TSPO+ and empty plasmid control cells, after gamma-irradiation to a preliminary dose range of 10, 100 and 5000 mGy. This was expressed as a percentage relative to the unirradiated controls (Figure 5.2), as well as by the frequency and extent of any changes (Figure 5.4). Gamma-irradiation significantly and consistently increased the production of both 24-HC and lathosterol in wild type cells after a dose of 10 mGy, suggestive of heightened cholesterol synthesis for conversion to 24-HC. The concentration increase observed at this dose for each replicate sample, when compared to each control, were between 206 and 757 % for lathosterol and between 12 and 226 % for 24-HC. The concentration range at 10 mGy was therefore distinct from unirradiated cells. Given the role of 24-HC in adaptive effects, likely as an LXR agonist, this sterol may form an integral part of the cell response to stress initiated by low-dose gamma-irradiation. Recovery of 25-HC was more variable and not overall significantly increased by irradiation, despite an increase from background levels in around 65 % of cases.
The production of 24-HC and lathosterol in wild type cells was reduced as dose increased, with only lathosterol yielding a significant and consistent increase after 100 mGy, ranging from 16 to 157 % for sample comparisons against each unirradiated control. Overall significant decreases were observed after 5000 mGy, which were less consistent, ranging from a 45 % decline from unirradiated controls to a 3 % increase. Except for the 100 mGy formation of lathosterol, overlap in the concentration range of each COP after irradiation with that for unirradiated cells prevents a discernible biodosimetric response. Correspondingly, 24-HC increased from control levels, together with 25-HC, in only about 65 % of cases after 100 mGy, and all metabolic sterol recoveries declined from controls after 5000 mGy with frequency of 65 to 80 %. This may suggest gamma-irradiation to adversely impact COP biosynthesis and/or the cell adaptive response as dose increases, consistent with existing thresholds (Section 1.3.3), or a sustained response that is independent of these sterols. An increase in the dose rate beyond 1 Gy/min in order to achieve doses $\geq 5000$ mGy may also have contributed to these observations.

Cell stress was evinced by an increase relative to controls, with an incidence of about 80 %, in all the cytotoxic C7 ring-oxidised COPs. In particular, 7-KC, the major autoxidative product, more than doubled in its production in almost two-thirds of cases. However, in the remaining cases a decrease in relative mass was observed, resulting in a response of ring-oxidised COPs that was varied and thus overlapped in concentration range between irradiated and unirradiated samples, leading to nonsignificant comparisons. The formation of autoxidative COPs trended toward a decline relative to controls after irradiation to higher doses, where cases of increased COP concentration were less frequent and/or extreme. This may support a sustained capacity for cell protective mechanisms at higher doses, or autophagic digestion of cytotoxic lipids, which may precede cell death (Yonekawa & Thorburn 2013).

The response of sterols to irradiation in TSPO+ cells was modulated by plasmid transfection. Analyte production was significantly reduced relative to unirradiated controls after 10 and/or 100 mGy in empty plasmid transfected cells, except for lathosterol after 100 mGy, which was significantly increased. Limited plasmid effects were previously reported by Liu et al. (2017) for the same cell lines when measuring markers of cell/mitochondrial function (discussed in Section 6.5). To account for these plasmid effects, the response of empty plasmid cells was subtracted from that of TSPO+ cells to find the net response from TSPO overexpression, relative to unirradiated controls and to wild type cells (Figure 5.3). This resulted in overall nonsignificant dose-responses for most analytes, as compared to the unirradiated controls, with some exceptions.
Lathosterol was significantly and consistently reduced in overall relative mass in plasmid-subtracted TSPO+ cells after 10 mGy, which declined further after 100 mGy, together with a significant and consistent reduction in the mass of 24-HC. This corresponded, for such low doses, to a significantly lower measured mass of these sterols in TSPO+ cells relative to unirradiated controls, as compared to that for wild type cells. This loss of 24-HC and lathosterol mass in irradiated TSPO+ cells is in contrast to the general elevation of both these sterols in unirradiated cells. Irradiation may therefore modify normal TSPO function, attributing to a change in the biodosimetric response that is dependent on the level of TSPO expression in the irradiated cells/tissues. Although more data is required to verify this hypothesis, it could be posited that cholesterol biosynthesis and subsequent generation of metabolites may be downregulated to avoid accumulation of cytotoxic COPs in the presence of elevated ROS, the formation of which is a prospective function of TSPO (Betlazar et al. 2016; Liu et al. 2017). However, 25-HC, which is synthesised by a microsomal enzyme like 24-HC (Diczfalusy 2013; Lund et al. 1999), exhibited more cases of upregulation after 10 mGy and was consistently, although not significantly, upregulated in response to a 100 mGy exposure. While the production of 25-HC can take place via autoxidation, its biological arrangement in cells makes this unlikely (Diczfalusy 2013). Therefore, this sterol may replace 24-HC in the adaptive cell response due to 24-HC downregulation, preparing cells for subsequent dose exposures or inducing controlled death of damaged cells, but requires further verification.

Overall 7α-HC concentrations in TSPO+ cells significantly and consistently increased relative to controls after 10 mGy, together with a rise in 7β-HC in 80% of cases, suggesting potential for heightened ROS effects in plasmid-subtracted TSPO+ cells. However, the change in 7α-HC production in TSPO+ cells after irradiation was nonsignificant when compared relative to that for wild type cells. Further, 7-KC was heightened in approximately 30% fewer cases after 10 mGy exposures in TSPO+ cells than wild type cells. As dose increased, the frequency of cases exhibiting increased recovery remained mostly consistent for all C7-oxidised COPs. Thus, the effect of TSPO during irradiation is unclear. It has been suggested that TSPO may confer its own cell protection against ROS (Carayon et al. 1996), such as those produced by irradiation, which may proceed without protective COPs. To further confound the issue, both 24-HC and lathosterol returned to within the variation of unirradiated controls after 5000 mGy (with elevated dose-rate). This yielded significantly greater overall relative levels of 24-HC and a potential for substantial, though highly variable levels of lathosterol, as compared to wild type cells. Gamma-irradiation to higher doses therefore appeared to cause failure of the low-dose
TSPO response, associated with a delayed response like that observed for wild type cells at lower doses. However, due to high levels of variation in measurements (Section 5.3.1) and empty plasmid effects, these results should be taken as indicative only and the experiments repeated under more ideal conditions (discussed in Section 6.5).

5.4.2 Sterol production in PBMCs

The isolated PBMC fraction can be expected to yield 95 ± 5 % mononucleocytes, comprised of about 85 % lymphocytes and 15 % monocytes (Bittersohl & Steimer 2016; GE Healthcare 2007). Monocytes account for most TSPO expression in PBMCs (Harberts et al. 2016), although a comparative study of TSPO expression between PBMCs and the TSPO+ Jurkat cells utilised by this study (Liu et al. 2017) has not been conducted. Human PBMCs were irradiated to a dose range of 1, 10, 100, 5000 and 10,000 mGy (Figure 5.5 and Figure 5.6). Evaluation of sterol production in the PBMCs suffered instrument failures (Section 6.5) and limitations due to quantitative sensitivity (Section 5.3.3) that resulted in sample loss. Nonetheless, from the data available it was apparent that the production of PBMC sterols in response to gamma-irradiation varied both within and between individuals. As previously discussed (Section 5.4), this variation between replicates may have arisen due to matrix interference or inherent variation of the methods employed (Section 5.3.1). However, the level of variation measured within PBMC sterols pooled across all sample donors (Figure 5.6) was greater than that for replicate Jurkat cells (Figure 5.3). This is exemplified by a lack of significant results for PBMCs, as compared to that for Jurkat cells, despite the frequent occurrence of large deviations from unirradiated controls. This result highlights the likely contribution of interindividual differences in the variation observed for PBMCs.

The pooled sterol quantities for all individuals comprised both increases and decreases in production after irradiation relative to unirradiated controls, which more frequently yielded changes greater than ± two-fold, than Jurkat cells. No significant overall change in the mass of 24-HC and lathosterol after irradiation was observed due to a near equal frequency of increased and decreased sterol production across all doses. This trended toward a higher number of reductions in the measured mass, particularly for 24-HC after 100 and 5000 mGy, and lathosterol after 10,000 mGy. Thus, while a typical change in the metabolic response to irradiation was apparent, the level of disparity prevents any consistent dose correlation. The variation between individuals may be due to differences in endogenous cholesterol or COP
levels, metabolic activity, or radiosensitivity (Huber et al. 1989; Stiles et al. 2014), despite an attempt to mitigate such variation by including only healthy individuals of the same age and sex.

Irrespective of a higher level of variation in the dose-response, PBMCs appeared much more sensitive to change in the level of COPs after gamma-irradiation than Jurkat cells. While the radiation response itself may be mostly inconsistent for both cell types, the observation of more samples exhibiting large (± two-fold) changes in the sterol profile for PBMCs than Jurkat cells is concordant with a greater radioresistance of Jurkat cells than PBMCs (Patwardhan et al. 2015; Turriziani et al. 2003). Indeed, a dose-response was more apparent for cytotoxic C7-oxidised COPs in PBMCs than Jurkat cells, for which the mass was progressively raised from that of controls, peaking at 100 mGy with a frequency of 85 to 90 %. This included around 60 to 70 % of replicates that increased by more than two-fold. However, a high level of variation and overlap in the concentration ranges at each dose prevented any biodosimetric relationship. The production of these COPs subsided at higher doses and dose-rate, which may reflect more rapid cell killing, preceding any substantial accumulation of cellular ROS, or adaptive cell changes, such as to clear cytotoxic compounds from the cell. To verify these observations, it is necessary to repeat these measurements using more sensitive methods with reduced matrix effects, together with a greater number of individuals (discussed in Section 6.5).

5.4.3 Application of sterols for biodosimetry

The large variation in the measured COP response after gamma-irradiation prevented any reliable determination of biodosimetric markers. This was attributed to both analytical and interindividual variation (Section 5.4), resulting in an overlap of concentration ranges between various doses, complicating any distinction between each dose based on the COP profile. This was especially true for PBMCs, which demonstrated a complete absence of significant effects for all sterols and doses (Section 5.4.2). Conversely, 24-HC and lathosterol isolated from Jurkat cells did demonstrate some consistency and significance in their production post-irradiation after doses of 10 and/or 100 mGy, which was dependent on TSPO expression (Section 5.4.1). Further, given a gamma-irradiation time for the lowest dose (10 mGy) of below 20 seconds (Section 5.2.3), changes to the sterol profile can be considered to occur immediately following exposure. This result highlights potential for metabolic COPs to act as biomarkers of gamma-radiation exposure, which may not have been reproduced in PBMCs due to differences in TSPO
expression, differences between individuals, and/or a greater susceptibility of PBMCs to matrix effects during analysis. Therefore, these influential factors require further exploration to fully appreciate any dose correlation, if one exists (discussed further in Sections 6.4 and 6.5).

5.5 Summary and conclusions

Gamma-irradiation of human Jurkat cells and PBMCs was shown to typically result in a change to the normal cellular production of various COPs and the cholesterol precursor, lathosterol. This effect was highly variable, which was likely contributed to by large measurement errors in the presence of cell matrix. However, the production of sterols in PBMCs was more highly varied than Jurkat cells, indicating interindividual differences to contribute to response variation, complicating the identification of suitable biodosimetric markers. The observed changes were also more substantial for PBMCs than Jurkat cells, particularly for the cytotoxic C7-oxidised COPs, which may infer a difference in radiosensitivity.

In Jurkat cells, both 24-HC and lathosterol appeared to be involved in the cell response to radiation-induced stress, which were significantly and consistently elevated in their recovered mass after a dose of 10 mGy to wild type cells. Lathosterol further exhibited a distinctive response after 100 mGy. Exposure to higher doses also demonstrated a dose relationship for these sterols, although inconsistency of such changes across all samples and/or overlap in the concentration ranges attained for lower doses prevented any dose correlation. Thus, while PBMCs may not have exhibited a discernible biodosimetric response using the current method, such consistent changes to Jurkat cells demonstrate potential for biodosimetry in different human cell types. However, these findings require verification using more sensitive and reproducible analytical methods.

Overexpression of TSPO in Jurkat cells after subtracting plasmid effects was found to modulate the dose-effect, resulting in downregulation of 24-HC and lathosterol at 10 and 100 mGy relative to unirradiated cells. These changes were significant, with reduced relative responses as compared to wild type cells. However, considering inherent method variation and that empty plasmid effects were often greater than that of the TSPO expression plasmid, this result should be interpreted with caution. Further, plasmid-subtracted TSPO+ cells demonstrated a contrasting response to wild type cells after 5000 mGy. Despite measurement errors, the significant effects that were consistently demonstrated for 24-HC and lathosterol, in both wild
type and TSPO+ cells, highlight a potential for biodosimetric variances in cells/tissues that differentially express TSPO. This implies a role for TSPO in modulating, rather than facilitating, the COP-driven cellular radiation response, although it remains unclear as to what effect.
Chapter 6: Conclusions and future directions
6.1 Introduction

Biological material located at a radiological crime scene may be exposed to substantial doses of ionising radiation; the resulting DNA damage may hinder the PCR required for amplification of the conventional autosomal forensic STRs (Section 1.2.6.2). Alternatively, mtDNA offers a greater chance for gaining identity information from degraded DNA due to its high copy number per cell (Section 1.2.2.2). However, ionising radiation can cause additional damage to mtDNA via localised oxidative reactions, potentially reducing its forensic value (Section 1.3.2.1). A comparative study on the degradation of both nuDNA and mtDNA extracted from the same cells after high doses of gamma-radiation has not previously been explored in the context of forensic relevance.

The same oxidative reactions that may lead to differences in nuDNA versus mtDNA integrity after high doses of gamma-radiation may also lead to cellular metabolic changes, which may generate biodosimetric markers after low dose exposures (Section 1.3.3.1). These biomarkers may assist radiation injury triage and correlate a person of interest with a radiological crime. Of particular interest are the oxysterols (COPs), derived from cholesterol oxidation, which have been implicated as biomarkers of cellular oxidative status, cell damage or death, as well as cell survival or adaptation to stress (Section 1.3.2.2.1). The formation of some COPs has been previously demonstrated to be modified by accidental gamma-radiation exposures, with the cellular effects of COPs in such oxidative conditions being the topic of prior investigations (Section 1.3.3.1). However, the endogenous production of COPs has not been performed for cells that have been exposed to incremental low doses of gamma-radiation, with the objective to identify any dose correlations for biodosimetric purpose and potential biological significance.

The sections that follow summarise the main conclusions from the development and validation of methodologies, and their application to the analysis of DNA degradation and cholesterol oxidation in gamma-irradiated cells. The limitations of these studies are discussed in the context of directions for future research.
6.2 Development of methodology for the analysis of mtDNA degradation

A series of singleplex qPCR assays using SYBR Green were developed as a means to quantify the extent of mtDNA degradation after gamma-irradiation (Chapter 2). The assays included quantification of a short (86 bp), medium (190 bp) and long (452 bp) amplicon, which were located within the 12S and 16S rRNA coding regions of the mtDNA genome. The ratios of longer to shorter targets informed indices of mtDNA integrity. A mtDNA standard was purified containing all three targets, from which unknown samples were quantified from the preparation of standard curves. Linearity ($R^2 \geq 0.999$) was achieved from $1 \times 10^8$ down to 10 copies, with accuracy and precision each within 10 % for most sample dilutions at 95 % confidence (Section 2.2.2). An IPC assay was also designed to verify the absence of PCR inhibitors and the validity of degradation assays. The purpose was to apply the integrity indices determined from these assays to the assessment of mtDNA degradation subsequent to gamma-irradiation. Such mtDNA integrity indices could then be compared to equivalent nuDNA integrity indices generated from peak height ratios of STR genotypes.

While the singleplex reactions were a simple and cost-effective means to assess mtDNA degradation, these reactions are laborious and consume more sample. For the precious samples likely to be encountered in a forensic or ancient DNA context, the combination of all three target and IPC assays into a hydrolysis probe-based multiplex PCR would be advantageous. This was the original intention at ACAD, where targets were selected within the homologous coding region to enable binding of a universal probe (TaqMan) between primer sites. Upon evaluation of sequence variation and homology within human mtDNA genomes, the selected sites were the only regions that satisfied these criteria (S.S. Tobe, personal communication, 30 March 2018). Thus, it was not possible to select similar targets within the control region (site of the HVRs) for the binding of universal probes necessary for multiplexing, due to its sequence polymorphic nature.

In lieu of directly assessing damage to the control region/HVRs, it was assumed that HVR damage could be extrapolated from degradation of the coding region targets; the assumption is that mtDNA damage occurs randomly and uniformly across the mtDNA genome. This approach should be considered with caution following gamma-irradiation, where the control region is more susceptible to oxidative damage than the coding region due to charge trapping at prevalent triple guanine (GGG) sites (Zhou et al. 2012). Therefore, it would be valuable to
select further targets of different length within the control region for singleplex reaction, such as with SYBR Green, particularly to incorporate primer sites for forensic HVR amplicons (Holland et al. 2011; Vallone et al. 2004; Wilson et al. 1995). For this purpose, primers applied for the amplification and sequencing of overlapping control region targets of various lengths may be appropriate (Berger & Parson 2009; Edson et al. 2004; Eichmann & Parson 2008; Gabriel et al. 2001).

For practicality, development of a quantitative universal probe-based (e.g. TaqMan) multiplex is desirable using the three coding region targets that were applied for singleplex reaction in this study. However, in either singleplex or multiplex format, the expected damage to HVRs/control region should be verified relative to the integrity indices determined from coding sites. This evaluation would benefit not only for gamma-radiation exposures that could be expected to increase oxidative stress, but also from the exposure of cellular mtDNA to varying degrees of humidity, endogenous/exogenous enzymatic digestion from decomposition processes, UV-irradiation and combinations of each. This would permit these assays to be applied for the evaluation of HVR damage from exposure to other such degrading sources, which are common to forensics and ancient DNA (Alaeddini et al. 2010).

### 6.3 Effects of gamma-irradiation on nuDNA versus mtDNA degradation and forensic genotyping

The degradation of DNA was investigated after exposure of human whole blood to high doses of gamma-radiation (Chapter 3). Doses from 1 to 50 kGy were selected based on previous examinations of STR genotyping efficacy over this dose range that demonstrated potential for significant damage (Abbondante 2009; Goodwin 2013). Thus, it is valuable to ascertain the comparative impact to mtDNA at such doses. Biological samples at a radiological crime scene could include both liquid and dried human tissue/blood matrices, where the former may comprise the predominant sample type, at least initially. Thus, both dried (dehydrated) and liquid (hydrated) blood were examined after gamma-irradiation for differential damage. It was conjectured that cell hydration would allow aqueous DNA to participate in more oxidation than dried cells due to secondary ROS reactions from water radiolysis and mitochondrial hyperfunction. Hence, mtDNA could be expected to receive greater damage than nuDNA where this takes place. To test this hypothesis, a comparison of nuDNA versus mtDNA was achieved.
by the evaluation of DNA integrity indices, which were determined from the peak heights of autosomal STR loci and the quantities of three mtDNA amplicons of differing size (Section 3.2.7). In addition, the experiments aimed to identify any dose thresholds or implications for the veracity of STR genotypes using the GlobalFiler PCR Amplification Kit, selected for containing all 17 current NCIDD and 20 current CODIS loci.

A progressive peak height reduction for high molecular weight amplicons (a characteristic of any degraded genotype) was evident as dose increased. However, gamma-radiation exposure did not greatly affect the ability to produce full STR genotypes, even at exposures as high as 50 kGy (Section 3.3.3). At this dose, allelic dropout below reportable thresholds occurred for liquid blood alone, with partial profiles due to the loss of < 20 % of alleles (above 225 bp). Liquid blood therefore exhibited greater degradation than dried blood at equivalent doses. Given the differential degradation arising from sample hydration status, an intermediate level of DNA damage could be expected for a liquid sample (blood/hydrated tissue) that is irradiated over the course of drying, which warrants further study. However, the genotyping thresholds applied were sufficient to prevent the false reporting of genotypes due to the dropout of single heterozygous alleles. Thus, lowering of stochastic thresholds cannot be recommended without stringent validation to ensure not to introduce such false calling of alleles.

Gamma-irradiation also resulted in a high prevalence of heterozygote peak imbalance, affecting 70 % of dried samples after a dose of 50 kGy and 80 to 90 % of liquid samples at and beyond 25 kGy (Section 3.3.3). The effect of gamma-radiation on heterozygote peak height imbalance has not been previously reported. The occurrence of peak height imbalances complicates profile interpretation where there are multiple contributors. Computational approaches for mixture deconvolution, such as STRmix (Bright et al. 2018; Bright et al. 2016), include models to account for varying degrees of degradation (Bright et al. 2013). Given heterozygote imbalances are common to degraded genotypes (Chung et al. 2004; Hansson et al. 2017), these computational models may therefore be sufficient for the interpretation of genotypes from gamma-irradiated DNA. However, future study should include an empirical evaluation to determine any dose threshold that may limit genotype interpretation software that are currently employed in forensic laboratories. This should entail an examination of single-source and mixed genotypes at various contributor ratios and degrees of degradation after high-dose (> 10 kGy) gamma-irradiation, including the use of different STR assays in current use.
While the GlobalFiler STR kit is currently applied for the amplification of all 20 CODIS loci for upload to criminal databases in the United States, an analysis of ionising radiation effects is required for other STR kits that are applied in forensic laboratories worldwide, such as the PowerPlex 21 System and NGM kits (Section 1.2.2.1). The degradation of PowerPlex 21 STRs after gamma-irradiation was previously investigated for low quantity buccal cell DNA that demonstrated significant degradation pre-irradiation, prepared both in suspension and dried (Goodwin 2013). Significant levels of allelic dropout were observed beyond 10 kGy, with an almost complete loss of reportable alleles after 50 kGy, highlighting a potential for substantial difference between genotyping assays and/or the type and initial quantity/quality of cell substrates.

While the DNA template applied in the present study was of high concentration (> 10 ng/µL), low template samples will elicit a greater degradative response, particularly in aqueous solution by increasing the molar ratio of water radiolytic ROS to DNA (Kong et al. 2010). Furthermore, modern kits are designed to withstand degradation by inclusion of amplicons below 200 bp; thus, differences in the level of observed degradation could be expected where locus size ranges are not consistent between genotyping kits. Current STR kits should therefore be directly compared for genotyping success from a range of commonly encountered cellular material (e.g. blood, saliva, hair, bone) of various quantity and quality, including samples that are environmentally and naturally degraded (e.g. sunlight exposure, decomposition processes) in addition to gamma-radiation exposure. Given overall genotyping success is a composite of all degradation processes, it would be advantageous to perform controlled studies, with a factorial design to determine contributions of each source to degradation. The combined degradation from each process would more accurately reflect the likelihood of obtaining an informative STR profile from a radiological crime scene, as well as the requirement for HVR sequencing.

Inhibition of the PCR required for DNA amplification is another common hindrance of forensic DNA analysis. The samples examined for ionising radiation-induced damage were suspected to be contaminated with low levels of PCR inhibitors, such as haem/haematin (from whole blood) and/or EDTA (from vacutainer tubes), discussed in Section 3.4. This is because lower doses (≤ 5 kGy for hydrated samples and ≤ 10 kGy for dehydrated samples) resulted in both an increase to peak heights and reduction in heterozygote imbalance, as compared to unirradiated genotypes (Sections 3.3.2 and 3.3.3). No other study of gamma-radiation STR effects at such doses has identified a similar phenomenon (Abbondante 2009; Monson et al. 2018), and thus it is doubtful that this is a natural cellular response. This can instead be explained by a greater susceptibility
of inhibitors to degradation from gamma-irradiation, than DNA. While blood samples were collected with EDTA tubes by both Abbondante (2009) and Monson et al. (2018), alternative DNA cleanup procedures were applied, which may have more efficiently removed inhibitors. The problem with this hypothesis was that the synthetic IPCs of the qPCR assays remained unaffected (Section 3.3.1). While the reaction chemistry and amplicon length are factors that can influence the effect of PCR inhibitors (Hall et al. 2013; Opel et al. 2010; Wang et al. 2008), this idea warrants verification. This can be achieved by spiking known concentrations (ranging from trace to high level) of common PCR inhibitors that are encountered in forensic samples (e.g. EDTA, haematin, melanin, calcium, humic acid) into purified DNA extracts and/or genomic control DNA prior to gamma-irradiation (≤ 5 kGy). Genotypes of samples with and without equivalent levels of spiked inhibitor can then be compared before and after irradiation.

The trends of STR genotypes were consistent with integrity indices, where dehydrated samples exhibited a radioprotective effect for both nuDNA and mtDNA in comparison to hydrated samples (Sections 3.3.4 and 3.3.5). While residual moisture may permit some effects of water radiolysis in dried samples, these results implicate a greater propensity for the generation of water radiolytic ROS in aqueous samples. This will contribute to greater generalised DNA damage in liquid blood than dried blood. Furthermore, nuDNA demonstrated greater radioresistance in comparison to mtDNA integrity indices (Section 3.3.6). Localised ROS formation due to mitochondrial hyperfunction may be one means by which mtDNA damage is amplified (Leach et al. 2001; Yamamori et al. 2012). However, dried samples also exhibited lower mtDNA integrity than nuDNA, suggestive of other mechanisms that may contribute to differential damage.

The radioprotection of nuDNA and/or radiosensitivity of mtDNA in desiccated cells could be afforded by (1) a complex innate structural arrangement (i.e. chromatin compaction, histones), despite mtDNA binding-proteins offering similar levels of protection (Falk et al. 2008; Gouliaeva et al. 2006; Takata et al. 2013); (2) a higher probability for mitochondrial than nuclear ionisation events as mitochondrial mass increases (Kam et al. 2013), which is recognised to form part of the upregulated mitochondrial radiation response (Yamamori et al. 2012); and/or (3) the presence of residual moisture, which preserves a level of cell functionality upon cellular desiccation that permits a mitochondrial ROS response, albeit at a reduced scale. The final point could be addressed by measuring the presence of ROS or oxidative markers in dried and liquid samples to determine the extent of difference, using methods such as those discussed in Section 6.5. Residual moisture content after sample air-drying could also be
determined by gravimetric analysis in comparison to equivalent samples completely void of moisture, such as by placement in a vacuum drying oven (De Valdez et al. 1985).

To further deconstruct these hypotheses, additional controls are necessary. This includes irradiation of naked (cell-free) DNA after both desiccation and dissolution in aqueous medium to evaluate cellular versus non-cellular effects. By applying such controls, Hall et al. (2014) revealed greater damage for dried cellular DNA than aqueous or dried naked DNA after UV-irradiation, which, while nonionising and not directly translatable to gamma-radiation, supports a level of continued damage after air-drying that is mediated by the cell. Controlled modulation of total mitochondrial content and number is possible by changes to the expression of mitochondrial DNA absence sensitive factor (MIDAS), which has been previously demonstrated by transfection into HeLa cell lines (Nakashima-Kamimura et al. 2005). This could be applied to further assess nuDNA versus mtDNA damage from ionising radiation in cell lines with differentially controlled total mitochondrial content. However, consideration must also be made of other cellular changes arising as a result of such transcriptional modifications that could influence DNA damage (e.g. MIDAS transfection also increases mitochondrial lipid content). Alternatively, nuDNA versus mtDNA damage to various cultured cells that are heterogeneous in total cellular mitochondrial number/content, e.g. HeLa, hepatocytes, astrocytes and neuronal cells (Collins et al. 2002; Robin & Wong 1988), could be compared after gamma-irradiation. This would require effects to be normalised against unirradiated cells of the same type before comparison to dissimilar cells.

The effect of mtDNA degradation, as measured by integrity indices determined from coding region targets, requires correlation with HVR sequencing capability (discussed in Section 6.2). It is likely that, despite higher levels of mtDNA than nuDNA damage, sufficient copies of intact mtDNA will be recovered, by virtue of the high copy number of mtDNA per cell, to enable sequence analysis of the HVRs. Gamma-irradiation of hairs up to 90 kGy did not impact HV1 and HV2 sequence analysis, nor did it impact STR analysis of bloodstains (Monson et al. 2018); however, HVR analysis should be evaluated in conjunction with STR analysis from the same cellular material. The limited impact of such doses on forensic DNA analysis, as supported by the findings of this thesis, accentuates the need to apply greater doses in order to identify a dose threshold, after which proceeding with such analyses would not be recommended. Monson et al. (2018) increased the dose in 10× increments, finding 900 kGy to substantially limit or completely prevent STR and HVR analyses, and therefore lesser
increments should be considered in order to identify a threshold dose within this range for which an informative result can still be attained.

Localised sample heating during irradiation may further contribute to DNA damage, due to cellular heat stress (Kantidze et al. 2016). This may be more substantial for aqueous than dried samples due to energy deposition in water molecules allowing for more efficient heating. While any heating effect could be similarly expected for samples irradiated by prolonged exposure in a radiological crime scene, this should be accounted for if the intention of future study is to explore mechanisms of nuDNA versus mtDNA damage due solely to radiation-induced processes. The efficiency of sample heating by gamma-radiation therefore requires investigation in order to determine the potential contribution of heat on DNA degradation. Such an evaluation could include intermittent temperature monitoring of mock samples (vials of water, glass slides) throughout irradiation, using identical dose-rates and final dose targets as applied to samples. This will direct the implementation of processes to mitigate these effects, such as the use of discontinuous sample irradiation or regular cooling intervals on ice.

Furthermore, drying of samples into the same vials as applied for liquid samples will prevent any change to attenuation of gamma-radiation due to the container type; however, this was considered to have had a negligible effect on the samples in this study (Section 3.4). While attenuation of gamma-radiation may be altered by glass composition and thickness, both the coverslips and vials were composed of borosilicate glass, with the difference in thickness between the coverslip and vial wall being considered insignificant. This is because the average distance between successive collisions (mean free path) for $^{60}$Co photons (~1000 keV; Section 1.1.2.1) that pass through borosilicate glass, containing lead additive, is in the range of several centimetres (El-Kameesy et al. 2018). A greater mean free path could be posited without the added density conferred by the presence of lead.

To mitigate against ongoing degradative cellular activity and ROS effects, samples were rapidly frozen post-irradiation. While this is a proven strategy for preserving DNA integrity (Hara et al. 2015), the potential for adverse post-irradiation cellular effects (Section 3.4) further accentuates the importance of sample storage conditions for DNA substrates exposed to ionising radiation. An examination of these effects should consider both dried and aqueous sample types, including cellular and non-cellular DNA, to discriminate effects due to radiation, aqueous environments, or ongoing cell function. Comparisons should be made between samples stored frozen (-20 °C and -80 °C), refrigerated (4 °C) and at ambient temperature (20-25 °C) for defined time periods,
similar to Abbondante (2009). This study would aim to direct future strategies for sample storage upon collection of irradiated biological evidence.

Finally, the same experiments described in this thesis could also be applied to alternate types of ionising radiation that may be encountered in a radiological crime scene, particularly alpha- and beta-radiation. This could take place at ANSTO, utilising their Small Tandem for Applied Research (STAR) and Australian National Tandem Research (ANTARES) particle accelerators and Activity Standards Laboratory. These facilities permit the acceleration of alpha particles that could be used to represent emission energies of common sources, such as $^{241}\text{Am}$, as well as enable exposure of samples to sealed beta-emitters, such as $^{90}\text{Sr}$. Ionising radiation of increasing LET could be expected to form a greater extent of clustered DNA lesions from direct interaction with DNA molecules (Section 1.3.2.1), with a reduced contribution from indirect/ROS mechanisms (Hirayama et al. 2009; Roots et al. 1985) and decline in sample penetrability (Veenema & Karam 2003).

STR genotyping with various kits (Identifiler Plus, Profiler Plus and/or Cofiler), as well as HVR sequencing, have demonstrated the possibility for high resistance to alpha- and beta-radiation up to the highest doses examined of 1200 kGy (Monson et al. 2018) and 51.6 kGy (Withrow et al. 2003), respectively. However, others have observed higher radiosensitivities, with degradation after 66 kGy of alpha-radiation (Abbondante 2009) and 50 kGy of beta-radiation (Shaw et al. 2008), although these were also associated with higher levels of degradation for gamma-irradiated genotypes. A consolidated study is therefore required using current STR kits to determine appropriate stochastic and dose thresholds for these radiation types, beyond which genotyping cannot be performed. Additionally, studies regarding the degradative effects of gamma-radiation on forensic DNA analysis have, thus far, focused on photon emission from a $^{60}\text{Co}$ source; a different pattern of degradation is possible for gamma-radiation sources of differing photon emission energies (e.g. $^{137}\text{Cs}$ and $^{192}\text{Ir}$), which merits further investigation.
6.4 Development of methodology for the quantitative analysis of cholesterol oxidation biomarkers and DNA co-extraction

A methodology for the quantification of several COPs, inclusive of 7α-HC, 7β-HC, 7-KC, 24-HC, 25-HC and 27-HC, as well as the cholesterol precursor, lathosterol, was developed (Chapter 4). The analysis by GC-MS required a large volume injection for the majority of derivatised COPs to permit MS detection/quantification (Section 4.3.1.2). GC-MS was selected for COPs analysis due to it being a well-established technique for this purpose, being considered the ‘gold standard’ method for such analyses, with derivatisation to TMS ethers being performed as a matter of routine (Dzeletovic et al. 1995; Freemantle et al. 2013; Griffiths et al. 2013; Guardiola et al. 2004; Hu et al. 2015; Kumar et al. 2011; Lai et al. 1995; Lee et al. 2008b; Riols & Bertrand-Michel 2018; Szucs et al. 2006). The extraction and analytical methods were evaluated by comparison of spiked calibration/recovery samples with and without the presence of cell culture medium (DMEM) and/or PBMCs. DMEM was applied for the growth and final suspension of Jurkat cells for irradiation experiments (Chapter 5) and thus, for consistency, was also used to suspend PBMCs for the same purpose. The DMEM, in the absence of cells, was spiked with cholesterol at a similar amount as expected for 5×10⁶ cells (in accordance with the cell number used for irradiation experiments) and subjected to the same sample workup procedure as applied for irradiated samples.

Quantification was achieved via the addition of an internal standard (19-HC) exhibiting similar physiochemical characteristics as the analytes of interest. Serially diluted analytes were normalised against a constant amount of internal standard for the preparation of calibration curves for quantification (Section 4.2.4.5). In this way, the internal standard is able to account for matrix effects and incomplete sample analyte recoveries, since it is subject to the same matrix and recovery losses that arise from the applied methods (i.e. extraction, purification, derivatisation).

The presence of DMEM matrix appeared to induce a decline in sensitivity following the preparation of calibration curves (Section 4.3.4.1.2), inducing a reduction in slope and linearity as compared to calibration curves prepared without DMEM (Section 4.3.4.1.1); this is discussed in Section 4.4.1. This matrix interference was evident as additional chromatographic peaks, retention time shifts, and changes to SIM ion ratios in the presence of DMEM extract (Sections 4.3.4.1 and 4.3.4.2). However, these effects may also reflect instrumental deterioration over time, since analysis of blank calibration samples was performed several months in advance of
matrix calibration samples. This is because the GC-MS system used in this study suffered from extended periods of instrument failure, including air leaks and rapid build-up of sample residue within the ion source (requiring regular cleaning), which could have impacted analytical efficiency over time. However, these issues were resolved prior to analysis of calibration curves and irradiated samples and did not appear to impact the back-calculated accuracies/precision of calibration (Section 4.3.4.1) or recovery results (Section 4.3.4.3).

DMEM was also detrimental to the recovery and analysis of co-extracted DNA (Section 4.3.2.3). While a solvent-based co-extraction procedure for the recovery of about 90% each of blank-spiked DNA and sterols was developed, less than 10 ng endogenous DNA was isolated per $1 \times 10^6$ PBMCs (quantified by Qubit fluorometry). The DMEM extract also inhibited PCR analysis. In light of these effects, consideration was made to transferring cells from growth medium into PBS immediately prior to irradiation. However, this would require cells to acclimate with the new medium, introducing another variable that may influence cellular effects. The replacement of DMEM with PBS prior to extraction was not possible since frozen cells would require thawing, similarly inducing cell stress. The replacement of DMEM entirely with PBS (by isolating PBMCs directly into PBS) may also induce stress during overnight storage, compromising functional cell integrity, prior to irradiation. The continued use of DMEM for PBMCs, as abovementioned, maintained consistency with Jurkat cells that were prepared for irradiation experiments.

The observations of PCR inhibition and interference of COP detection signals could indicate co-extraction of aqueous and organic components of DMEM that are averse to analyses. The components of DMEM, being a water-based culture medium, are water-soluble and could be expected to be recovered into the aqueous phase. This includes inorganic salts, amino acids, vitamins, glucose, pyruvic acid, buffering agent (4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid, HEPES) and pH indicator (phenol red). Upon extraction in chloroform/methanol, certain components may have some affinity for the organic phase, resulting in complete or partial organic partitioning of certain components, such as hydrophobic amino acids (Vertommen et al. 2010) or HEPES buffer (Taha & Coutinho 2016).

Due to the mixed-mode nature of the stationary phase applied for SPE (Section 1.4.2.2), it is possible that any such hydrophobic groups could be retained by the nonpolar portions of the sorbent; further, the polarity of HEPES, conferred by two hydroxyl and carbonyl groups, enable affinity for the polar portions of the sorbent. Deprotonation of amino acids (carboxylic acids)
and HEPES (hydroxyl moieties) under high pH allows further retention of these groups (by the anion exchanger) during the initial alkaline solvent wash, which are disrupted by the final acidic wash to enable possible co-elution in the nonpolar and slightly polar COP elution solvents. The presence of certain buffer systems, including HEPES, has been demonstrated to suppress ionisation efficiency by electrospray ionisation (ESI), as applied to LC-MS (Castrillo et al. 2003). While dissimilar to EI, the effects on ESI highlight potential for matrix effects to impact MS techniques due to components of cell culture media. Further evaluation is required of blank DMEM extracts to determine the likely co-extracted components.

Despite containing the same general constituents (Arora 2013), use of an alternative cell medium may mitigate these effects if modified to exclude possible problem components (e.g. HEPES). Additionally, the extent of qPCR inhibition was shown to differ between various enrichment broths (Hyeon et al. 2010); hence, different culture media, such as Roswell Park Memorial Institute (RPMI) medium that is recommended for Jurkat cells (Arora 2013), may similarly alleviate inhibition by inclusion of differing component concentrations. However, studies that have utilised DMEM prior to extraction for COPs analysis by GC-EI-MS or DNA analysis by PCR have not reported any such effects (Frolov et al. 2003; Fu et al. 2001; Giengkam et al. 2015). Although, given evidence for the contrary in this thesis, future analysis may benefit from an enhanced purification approach to better minimise the recovery of matrix interferents.

Most DNA and sterol loss was observed at the stage of sample purification by ethanol precipitation or SPE, respectively, at around 50 % for DNA spikes (Section 4.3.2.2) and 20-50 % for sterol spikes (Section 4.3.4.3). Hence, to improve the co-extraction technique, further optimisation should focus on these purification procedures. An improved SPE procedure for almost complete COP recovery is possible with aminopropyl columns, eluting nonpolar compounds with hexane and polar sterols with a mixture of chloroform and methanol (McDonald et al. 2012). For DNA cleanup from hydrophilic PCR inhibitors that may ethanol precipitate with DNA, the use of alternative purification approaches may be more suitable, such as ultrafiltration, the application of silica resins, or a combination of both (Alonso et al. 2001). Modification to the ethanol precipitation procedure may also facilitate improved recoveries, including the use of room temperature incubations (reducing solution viscosity) or DNA carrier, such as glycogen or linear polyacrylamide (Fregel et al. 2010; Gaillard & Strauss 1990; Zeugin & Hartley 1985). The DNA losses after ethanol precipitation were consistent with observations by Zeugin and Hartley (1985) for DNA amounts below 100 ng, which could be improved by
centrifugation for minimally 30 minutes. Alternatively, an aliquot of each sample could be collected for DNA extraction, separate from the solvent-based lipid extraction, using a commercial kit such as that employed in Chapter 3.

Further problems were encountered during the derivatisation of sterols to TMS ethers. Thermal decomposition of 7α-HC, 7-KC and 25-HC necessitated a large reagent excess (> 200×) in the presence of pyridine base catalyst during incubation at 80 °C for 2 hours, as well as 40 % (v/v) reagent in the final injection solvent, to achieve complete derivatisation (Section 4.3.1.1). No other study encountered over the course of this method development utilised such extreme conditions for derivatisation of these same analytes. Using the same reagents (BSTFA, 1 % v/v TMCS), complete derivatisation of the sterically hindered 25-hydroxyl moiety was possible in 1 hour at 60 °C or 30 minutes at room temperature with the inclusion of pyridine (Guardiola et al. 2004; Lai et al. 1995). Hence, the requirement for such extreme conditions during this study could not be explained, considering the techniques employed are not unfamiliar to COPs analysis (discussed in Section 4.4.1). Consequently, the reactions were time-consuming and costly, owing to the large volumes of silylation reagent required per reaction (200 µL), and compelled the use of an equal volume of pyridine, a highly volatile and toxic solvent. The lengthy and harsh silylation procedure may be improved by the use of a stronger reagent blend, such as BSA/TMSI/TMCS (3:3:2 v/v/v) (Guardiola et al. 2004), but can be avoided by the use of alternative analytical techniques.

Negative chemical ionisation (NCI) was applied for the mass selective detection of cholesterol tracers after GC by Östlund Jr. et al. (1996). Pentafluorobenzoyl (PFB) ester derivatives were prepared from 1 µg to 1 mg of cholesterol, dissolved in 200 µL toluene, in the presence of 5 µL PFB chloride and 20 µL pyridine at room temperature for 10 minutes. The GC-NCI-MS technique also featured improved sensitivity to traditional GC-EI-MS, permitting detection of the derivatised product as low as 1 femtomole (f mol), or 580 fg (Östlund Jr. et al. 1996). A potential limitation of NCI is that it is a soft ionisation approach, generating minimal to no fragmentation ions that ordinarily (by EI) allow for the unique characterisation of COPs with the same molecular ion. This could be overcome by adjusting the GC temperature program (e.g. reduced ramp rate) to ensure complete analyte separation into different SIM windows for the targeted and quantitative analysis of COPs.

Derivatisation may be circumvented altogether by the use of LC techniques, since heat protection of thermolabile COPs is not required. Coupling of LC to triple quadrupole tandem
MS is necessitated to match the sensitivity and selectivity of GC-MS ionisation modes (Krone et al. 2010; McDonald et al. 2012; Narayanaswamy et al. 2015; Shan et al. 2003). McDonald et al. (2012) applied this technique with ESI for polar COPs or atmospheric pressure chemical ionisation (APCI) for neutral sterols, both soft ionisation approaches. A triple quadrupole was operated with collision-induced dissociation (CID) to increase analyte fragmentation and signal intensities. Their method, using these approaches, was sensitive to injection of 0.05 ng and quantitative to 1 ng/mL, which is a similar level of sensitivity attainable by GC-EI-MS (McDonald et al. 2012). However, McDonald et al. (2012) were unable to resolve certain sterols that were resolvable by GC, including 7α-HC and 7β-HC, as well as cholesterol and lathosterol.

The weak ionisation efficiency of sterols by desorption ionisation modes, such as ESI and APCI, necessitates derivatisation to improve detection signals and resolving power (Griffiths et al. 2016; Krone et al. 2010). Many methods exist for the derivatisation of COPs for LC-MS analysis, most popular being the Girard hydrazones, capable of quantification at the level of 1 to 0.03 ng/mL (Griffiths et al. 2016). Thus, while demonstrating potential for higher sensitivities than GC-MS with an appropriate instrumental setup, these methods introduce lengthy steps akin to GC derivatisation, inclusive of the use of pyridine and/or other toxic solvents. However, the use of alternative derivatisation, chromatographic, and ionisation approaches required for COPs analysis by LC-MS (rather than GC-MS) could also be expected to modify associated matrix effects (Silvestro et al. 2013). This may be due to differences in the compatibility of interferents with the derivatisation or MS ionisation techniques of GC-MS versus LC-MS, reducing the ionisation efficiency of interfering compounds as compared to analytes of interest. Separation of COPs by LC-MS commonly takes place with reverse-phase octadecylsilica (C$_{18}$) columns (Guardiola et al. 2004; Jiang et al. 2011; Karu et al. 2007; McDonald et al. 2012; Meljon et al. 2012; Narayanaswamy et al. 2015), which may better resolve analytes of interest from the matrix interferents encountered in this study. In this way, a reduction of matrix effects leading to improved sensitivities and quantitative reproducibility may be possible by LC-MS and should be considered for future COPs analysis. To verify this conjecture, a comparison of the matrix effects generated under the conditions applied in this study for GC-MS versus LC-MS is required.
6.5 Formation of cholesterol oxidation biomarkers in human cells after gamma-irradiation

The production of sterols was measured in gamma-irradiated PBMCs (predominantly lymphocytes), isolated from human whole blood, and Jurkat cell lines (immature lymphocytes), inclusive of wild type, TSPO+ and empty plasmid control cells (Chapter 5). Low doses were selected (1 to 5000 mGy for Jurkat cells and up to 10,000 mGy for PBMCs) that could be expected to elicit a physiological cellular response, such as adaptive or cytotoxic effects (Section 1.3.3). TSPO has been implicated in radiation-induced effects including apoptosis and ROS formation, as well as cholesterol transport (Section 5.4). Thus, it was hypothesised that TSPO overexpression would exaggerate the dose-response to assist the identification of biodosimetric markers, as well as elucidate a possible involvement of COPs in the cellular effects mediated by TSPO. While matrix effects in the developed method appeared to account for a high level of error in the quantification approach (based on the variation of unirradiated controls; Sections 5.3.1 and 5.4), some consistent and significant changes to sterol production were identified.

Control cells transfected with empty plasmid demonstrated a significant reduction in the quantities of most sterols after irradiation, indicating that the vector itself was able to modulate the radiation response as measured by COPs (Section 5.3.2). The tendency for empty plasmid control cells to downregulate COP formation may be indicative of competition for energy or replication machinery (e.g. polymerase, cofactors) between the vector and host cell genome. However, no significant differences between wild type and empty plasmid cells were identified in the original study by Liu et al. (2017), which examined mitochondrial ETC gene expression and adenosine triphosphate (ATP) production, cell proliferation and motility, as well as potassium ion currents. Liu et al. (2017) noted that outward currents of empty plasmid cells peaked at -20 millivolts (mV), while wild type and TSPO+ cells peaked at 0 mV, which may substantiate the potential for differences introduced by the empty plasmid. A correction for such plasmid effects could be applied in the analysis of TSPO overexpression by subtracting the plasmid response from that of TSPO+ cells.

Overexpression of TSPO as compared to wild type Jurkat cells (containing negligible TSPO expression) caused a significant and consistent downregulation of 24-HC and lathosterol at doses of 10 and/or 100 mGy, relative to unirradiated controls (Section 5.3.2). This trended toward an elevated response as dose increased to 5000 mGy. This effect continued after
subtraction of the background plasmid effects. In contrast, a significant and consistent hyperproduction of these same analytes at such doses was observed when isolated from wild type Jurkat cells, with a tendency for the response of these analytes to decline at the highest dose. Thus, these metabolic sterols displayed potential for a role in the radiation-induced cellular response that could be applied for biodosimetry, which is dependent on the TSPO expression levels of the irradiated cells/tissues. While these findings should be considered with caution due to the plasmid effects and method variation, the significant and contrasting modification of the same sterols in wild type and TSPO+ cells indicate non-chance effects due to radiation. Thus, TSPO overexpression was demonstrated to modify the response of wild type cells (downregulating sterols that were upregulated and vice versa), rather than amplifying all responses as expected. Further evaluation is necessary to determine the extent of TSPO overexpression in the Jurkat cells employed by this study as compared to PBMC (monocyte/lymphocyte) expression levels, such as by determination of TSPO density in each cell type (Canat et al. 1993; Rochard et al. 2004).

The concentrations of remaining analytes were too highly varied between replicates and/or individuals to identify any significant trends. However, gamma-irradiation frequently demonstrated a capacity to induce change in the concentration of biomarkers that was greater than two-fold (Sections 5.3.2 and 5.3.3). This included observations of both increases and decreases in sterol concentration after the same dose, as compared to unirradiated cells. This was particularly evident for pooled PBMC sterol isolates collected from multiple individuals, emphasising the impact of interindividual differences on biodosimetric potential. However, the observation of within-sample variation of unirradiated replicates (Section 5.3.1) for both cell types may also suggest that method variation contributed to inconsistent results.

The presence of DMEM culture medium had an adverse effect on the calibration curves used for quantification (discussed in Section 6.4). This may impact the quantitative reproducibility of analyses. As discussed, a more comprehensive evaluation of the quantitative limits introduced by DMEM should be considered before further use of this method, as well as the use of an alternative culture medium, cell preparation, or purification method to improve calibration results. While these matrix effects did not appear to impact accuracies or precision based on spiking of analytes into cell-free media or cell matrices (Section 4.3.4), a large variation in sterol concentration for unirradiated cell replicates was apparent (Section 5.3.1). This suggested the measurement of trace endogenous sterols to be influenced by cell matrix. Due to time and economic constraints, appropriate experiments using cell matrix were unable to be performed to
more accurately determine the method limitations. This should ideally take place using stable isotope labelled analogues of each analyte, such as with deuterium ($^2$H) or carbon-13 ($^{13}$C), which can be spiked in the presence of cells at equivalent concentration to endogenous COPs (Shaw et al. 2014; Whiting et al. 2001). A decline in the performance of the GC-MS instrument could also have been a potential contributor to within-sample variation and sensitivity loss due to matrix effects.

The quantitative sensitivity limits prevented analysis of 3 to 21% of PBMC samples for analytes other than 25-HC, with concentrations below the LOQ in 50% of samples, and 27-HC, which was below the LOQ in 86% of samples (Section 5.3.3). Thus, 25-HC and 27-HC could not be reliably examined in PBMCs. Furthermore, 27-HC was not quantifiable in any Jurkat cells (Section 5.3.2). Considering the effects of irradiation and TSPO on both lathosterol and 24-HC, two metabolic-derived sterols, it can be posited that similar effects could be observed for other metabolic sterols, such as 25-HC and 27-HC that could not be appropriately analysed. For this reason, the method sensitivity was a substantial limitation in this study. Future evaluation of these biomarkers at the trace levels found in human cells must therefore apply more sensitive methods. This could include a GC-NCI-MS approach, as detailed by Ostlund Jr. et al. (1996), with potential for sensitivity in the femtomole range, or a LC system with triple quadrupole tandem MS, with possible sensitivity of derivatised COPs below 1 pg/µL (discussed in Section 6.4).

Considering a more sensitive analytical method, the experiments require repetition. To confirm interindividual variability of PBMC sterols, cells should be collected from a larger number of individuals. A larger number of replicate samples should also be prepared to increase the statistical power of analyses, enough to account for any inadvertent sample loss. A wider range of metabolic and autoxidative COPs could be examined, such as 4β-HC, 20-HC, 22-HC, 24,25-EC, 5,6-EC, and 3β,5α,6β-CT, each exerting cytotoxic or cell protective functions (Gill et al. 2008; Kulig et al. 2016; Murphy & Johnson 2008). For biodosimetric purpose, measurement of COPs extracted from whole blood or plasma samples after gamma-irradiation would be beneficial, which is in accordance with previous methods for such biodosimetry measurements (Bertho et al. 2009; Bertho et al. 2008). However, such measurements require an understanding of the time course for any biological change in the sterol profile (i.e. how rapidly after exposure do such changes arise and how long do they persist?), as well as the influence of potential confounding factors resulting from the diverse physiological and pathological roles of COPs.
Significant changes to the sterol profile can occur within seconds of gamma-radiation exposure (Section 5.4.3). These changes could then persist for several weeks after initial exposure (Bertho et al. 2009; Bertho et al. 2008). However, the sterol response is unlikely to be stable over this time; radiation biomarkers traditionally demonstrate a delayed onset or progressive rise in their effect, leading to significant changes post-irradiation after 24 to 72 hours, or longer (1-2 weeks), before slowly returning to basal levels (Rana et al. 2010; Singh et al. 2016). Therefore, future study of sterol biomarkers should consider periodic examination of samples that are incubated for several weeks under physiological conditions post-irradiation. Potential confounding factors should also be explored, which may alter the radiation-induced biomarker response and explain interindividual variation of results (Cruz-Garcia et al. 2018; Paul & Amundson 2008). Factors that may alter COP formation include individual metabolic rate (Guillemot-Legris et al. 2016; Hasson et al. 2011), endogenous and exogenous (dietary) cholesterol (Axelson & Larsson 1996; Sozen et al. 2018), lifestyle (e.g. smoking status, exercise) (Ferreira et al. 2017; Linseisen et al. 2002; Mol et al. 1997), and various disease states associated with COPs and/or oxidative stress (Poli et al. 2013). To investigate these combined effects, gamma-irradiation could be performed on blood cells or plasma collected from individuals with known variations in such potential confounding factors.

In order to better elucidate the cellular effects of COPs in the radiation response, direct measurement of cell viability and adaptation mechanisms (e.g. antioxidants, DNA repair) is required. Cell survival/apoptosis could be monitored by cell counting of irradiated aliquots upon viability staining (e.g. Trypan blue, Annexin V/propidium iodide) immediately after irradiation, which could be aided by flow cytometry (Oancea et al. 2006; Rieger et al. 2011). A simple measure of total antioxidant capacity can be achieved by the use of spectrophotometric or fluorometric assays, including ROS scavenging or redox potential assays (Kambayashi et al. 2009; Shahidi & Zhong 2015). A comprehensive analysis of cell viability, oxidative stress and antioxidant activity, as well as DNA damage signalling and repair, can be achieved via the use of commercial gene expression assays (Lu et al. 2017; Souliotis et al. 2016; Sun et al. 2015; Tahmasbpour et al. 2016).

To further evaluate the cell response to ionising-radiation, additional markers could be assayed, complimentary to COPs. This could include direct measurement of ROS using spectrophotometric or chemiluminescent detection methods, or the measurement of reliable ‘gold standard’ products of ROS interaction (Griendling et al. 2016; Pavelescu 2015; Woolley et al. 2013). The latter includes the DNA damage marker, 8-oxoG (Section 1.3.2.1), which can
be analysed by Southern blotting, qPCR or comet assays after inducing fragmentation of oxidised sites; by immunoassays; or by direct analysis via GC-MS or LC-MS (Griendling et al. 2016). The lipid peroxidation products termed F2-isoprostanes (Section 1.3.2.2), particularly 8-isoprostaglandin-F2α, are also reliable markers of cellular oxidative status, which can be measured by GC-MS or LC-MS (Janicka et al. 2013; Lee et al. 2008b; Tsikas & Suchy 2016; Xiao et al. 2016). Together, this highlights a practical application for a co-extraction procedure for both DNA and lipids; the development of such a procedure, with focus on a superior purification technique, should therefore be continued as per previous discussion (Section 6.4).

Finally, considering more sensitive methods than those applied in this thesis (discussed in Section 6.4), an examination of these biomarkers and cellular effects would additionally be of value for common ionising-radiation sources other than 60Co. This includes gamma-emitters of varied emission energies (e.g. 137Cs and 192Ir), as well as alpha-emitters (e.g. 241Am, 252Cf, 238Pu) and beta-emitters (e.g. 90Sr). These sources are listed as those most likely to be encountered in a radiological terrorism scenario (Ferguson et al. 2003). As discussed previously, such irradiation experiments could be performed using ANSTO facilities (Section 6.3). An altered oxidative stress response as compared to gamma-radiation could be expected with increasing LET (Hirayama et al. 2009; Roots et al. 1985), which may therefore produce varied biodosimetric and cellular effects that deserve consideration.

6.6 Final summary and conclusions

This thesis investigated the impact of gamma-radiation exposure of human cells upon DNA degradation, and the production of cholesterol oxidation biomarkers (oxysterols, COPs), in the context of the forensic analysis of a radiological crime. The study intended to demonstrate the potential implications of high-dose gamma-irradiation for forensic STR analysis, as well as investigate the potential for greater comparative damage to mtDNA targets dependent on cellular functional integrity. Furthermore, the study aimed to quantify COPs in human cells as potential biodosimetric markers after low doses of gamma-radiation, which have been implicated as informative markers of cellular oxidative damage, metabolic adaptation and the cell survival/death response. Thus, modulation of COP formation was additionally evaluated with the intent to elucidate possible radiation-induced cellular changes.
Developmental work established a novel method to quantitatively assess mtDNA degradation by qPCR. This assay was applied together with autosomal STR analysis, revealing high doses (> 10 kGy) of gamma-radiation to induce a greater extent of overall DNA damage in liquid blood as compared to dried blood. However, a minimal effect on GlobalFiler STR genotypes was evident up to the highest dose examined of 50 kGy. Thus, in a radiological crime scene, successful STR genotypes can be expected from biological evidence exposed to substantial doses of gamma-radiation. Furthermore, this study demonstrated the aptness of genotyping peak amplitude thresholds to prevent the false reporting of alleles and is the first to report the impact of heterozygote peak imbalance on STR genotypes generated from gamma-irradiated DNA. No dose threshold could be identified, within the doses examined, that reduced STR genotyping efficacy to warrant HVR sequencing of mtDNA. In the event that HVR sequencing is required due to STR degradation, the forensic value of the HVRs is questionable due to mtDNA demonstrating greater radiosensitivity than nuDNA at equivalent doses of gamma-radiation.

A quantitative approach for the extraction, purification and analysis of selected sterols/COPs by GC-MS was also developed. Sensitivity limitations, coupled with a high level of variation in the sterol response for both PBMCs and Jurkat cell lines after low doses (up to 10 Gy) of gamma-radiation, hindered the identification of suitable biodosimetric markers. In particular, PBMCs demonstrated increased variation due to differences between individuals and/or a greater susceptibility to analytical matrix effects. These method limitations served to exemplify the importance for an analytical method to minimise matrix interference, while maximising sensitivity and reproducibility for biomarker monitoring in a radiation incident. However, despite these shortcomings, this study demonstrated a significant and consistent hyperproduction of 24-HC and lathosterol after 10 and/or 100 mGy, declining at 5000 mGy. After accounting for changes induced by plasmid transfection, these effects were shown to be modified by TSPO overexpression, resulting in an opposing and significant response of these same sterols at equivalent doses. These sterols may therefore function in the radiation-induced cell response. Furthermore, the potential for TSPO expression level to alter the biodosimetric response of gamma-irradiated cells/tissues has been demonstrated for the first time, laying foundation for future investigation using more sensitive and reproducible methods.
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