Characterisation of lupin-derived lupeol with a metabolomics study of the impact and potential neuroprotection of lupeol

by

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Declaration

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

Michael James Pilkington

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Abstract

Lupins are an agriculturally significant, dietary legume expressing multiple pharmacologically active minor components including the triterpenoid lupeol. However, there is currently a lack of knowledge regarding the abundance of lupeol in lupin seeds and the relative abundances of lupeol in seed hulls, kernels and embryos. In this work, lupeol in *L. angustifolius* seeds and seed fractions was characterised using GC-MS QQQ analysis of lupin seed matrix extracted using a novel application of the QuEChERS approach, a pesticide residue extraction methodology. Lupeol was significantly more abundant in isolated seed hulls than whole seeds or germ and was absent in seed kernels, indicating a potential localisation of lupeol to the hull of lupin seeds. Despite demonstrating various pharmacological activities and bioprotective functions, neuroprotective properties of lupeol have not been investigated. Cell concentration and a metabolomics approach were utilised to examine the response of rat cortical neuronal cell cultures to lupeol (10 µM) and investigate potential neuroprotection against an acute dose of the neurotoxin caffeine (1 mM). Lupeol, caffeine and co-administration of lupeol with caffeine had no significant impact on cell concentration after 24 hours despite caffeine and co-administration treatment visibly inducing a toxic response. Metabolomics investigations illustrated lupeol minimally impacted the metabolite profiles of cell cultures after 4 and 24 hours, with the exception of an intracellular reduction in tryptamine, signifying a potential neuromodulatory effect. Caffeine generally reduced intracellular metabolites including most amino acids and carbohydrates, in addition to GABA and tryptamine, whilst extracellular metabolites (notably fructose) largely increased in abundance, indicating increased catabolism of metabolites for ATP production or increased carbohydrate efflux, potentially resulting from cell membrane damage. Co-administration with lupeol and caffeine replicated these changes, with significantly greater reductions in aspartate, ribitol and ribose relative to treatment with caffeine only, while extracellular metabolite levels were not significantly different. Lupeol therefore appeared to demonstrate no toxicity in isolation but failed to provide neuroprotection against caffeine toxicity and may have potentiated the intracellular toxicity of caffeine by increasing the catabolism or utilisation of amino acids and carbohydrates.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>B50</td>
<td>B50 Rat Neuronal Cell Line</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DC</td>
<td>Direct Current</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DXM</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EI</td>
<td>Electron Ionisation</td>
</tr>
<tr>
<td>EIC</td>
<td>Extracted Ion Chromatogram</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Reduced Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric Acid</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatograph</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography – Mass Spectrometry</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced Glutathione</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes Simplex Virus Type I</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol (1,4,5)-Triphosphate</td>
</tr>
<tr>
<td>ISTD</td>
<td>Internal Standard</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid Chromatography – Mass Spectrometry</td>
</tr>
<tr>
<td>M⁺</td>
<td>Molecular Ion</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-Charge Ratio</td>
</tr>
<tr>
<td>MIZ</td>
<td>Mean Inhibitory Zone</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometer</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>MSTFA</td>
<td>N-Methyl-N-(Trimethylsilyl) Trifluoroacetamide</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PC</td>
<td>Principal Component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>PROT</td>
<td>Proline Transporter</td>
</tr>
<tr>
<td>Q₁</td>
<td>Quadrupole One</td>
</tr>
<tr>
<td>Q₂</td>
<td>Quadrupole Two</td>
</tr>
<tr>
<td>Q₃</td>
<td>Quadrupole Three</td>
</tr>
<tr>
<td>QMF</td>
<td>Quadrupole Mass Filter</td>
</tr>
<tr>
<td>QQQ</td>
<td>Triple Quadrupole</td>
</tr>
<tr>
<td>QTOF</td>
<td>Quadrupole Time-of-Flight</td>
</tr>
<tr>
<td>QuEChERS</td>
<td>Quick, Easy, Cheap, Effective, Rugged and Safe</td>
</tr>
<tr>
<td>RSP</td>
<td>Ribose-5-Phosphate</td>
</tr>
<tr>
<td>RF</td>
<td>Radio Frequency</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
</tr>
<tr>
<td>RT</td>
<td>Retention Time</td>
</tr>
<tr>
<td>TIC</td>
<td>Total Ion Chromatogram</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-Flight</td>
</tr>
<tr>
<td>UHP</td>
<td>Ultra High Purity</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume-Volume</td>
</tr>
</tbody>
</table>
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Thank you to A/Prof. Robert Trengove, A/Prof. Ian Mullaney and Dr. Garth Maker for their supervision, guidance and advice throughout this project.

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1. Introduction
1.1 Lupins

Lupins are members of the *Lupinus* genus, comprising over 500 species of perennial and annual herbaceous angiosperms (Kurlovich *et al.*, 2002; Domaica, 2010). Although major centres of lupin diversity have developed in the Mediterranean Basin, North America and South America, the genus is globally distributed across multiple continents including Asia, Africa, Europe and Australia (Kurlovich *et al.*, 2002). Such distribution is primarily the result of limited soil and climate requirements, enabling growth in poor, sandy soils with a pH of 5.0 to 7.0 and in temperatures between -9 and 29°C (Domaica, 2010).

Lupins grow up to 4 m in height and display 3 or 4 orders of lateral branching, each with terminal inflorescence as flower spikes (racemes). Lupins express fruit as a pod that encapsulates 2 to 7 seeds, which utilise the pod wall as an energy source during growth (New South Wales, 2011). Seeds display a variety of morphologies but are typically 8 to 14 mm wide with white, grey or brown colouration, smooth, fine-meshed, speckled or mottled surfaces and may be flattened, ovular or spherical in shape (Kurlovich *et al.*, 2002; Faest, 2010). Structurally, lupin seeds are composed of a fibrous outer seed coat (hull or testa comprising three cell layers), housing a protein-rich kernel (composed of two cotyledons), which provide a nutrient supply for an internally developing embryo (germ) (Miao *et al.*, 2001; Domaica, 2010). Examples of morphological diversity and the anatomical structure of lupin seeds are illustrated in Figure 1.1.

![Figure 1.1](image)

**Figure 1.1:** (A) Diversity of lupin seed morphology illustrated by *L. angustifolius* (left) and *L. albus* (right); (B) Representative lupin seed structure illustrating outer seed hull (testa), kernel (cotyledon) and developing embryo (shoot apex, hypocotyl and radicle) (retrieved from New South Wales, 2011).
1.1.1 Australian Lupin Production

Lupins are a commercially cultivated pulse crop in more than 20 countries including Australia, Chile, France, Poland, Russia and South Africa (Brand and Brandt, 2000, Erbas et al., 2005; Sweetingham and Kingwell, 2008). Australia is currently the largest global supplier of lupins, primarily harvesting *L. angustifolius* (narrow-leaf or Australian sweet lupin) and to a lesser extent, *L. albus* (white lupin) (Australian Bureau of Agricultural and Resource Economics, 2007; Sweetingham and Kingwell, 2008). Between 1997 and 2007, Australia was responsible for approximately 85% of global lupin production, generating an annual average of 1.2 million tonnes of lupins. Of this, Western Australia was the primary source of the commodity and was responsible for approximately 80% of total Australian-grown lupins produced in the same period. The global dominance of Australia in the lupin industry is primarily due to the ability of lupins to flourish in the sandy and acidic soils that are prevalent throughout the state (Australian Bureau of Agricultural and Resource Economics, 2007). In 2011, annual lupin production was estimated to be worth approximately $65 million (Australia. Department of Agriculture, Fisheries and Forestry, 2012).

1.1.2 Applications of Lupins

Despite the large number of lupin species known to exist, few are of current agronomic interest, a consequence of potentially toxic quinolizidine (lupin) alkaloids, which are endogenously synthesised in all lupin plant organs as secondary metabolites for predatory defence (Wink et al., 1995; Reinhard et al., 2006; Lee et al., 2007a). When consumed, lupin alkaloids such as lupanine, sparteine and angustifoline impart a bitter taste on the palate of predators and exert varying toxicodynamics including teratogenicity in livestock (such as crooked calf disease in cattle) and neurological symptoms in mammals including nausea, respiratory arrest, visual disturbances, ataxia, trembling, convulsion and coma (Australia New Zealand Food Authority, 2001; Lee et al., 2007b). Lupin alkaloid toxicity results from interactions of the alkaloids with nicotinic and muscarinic acetylcholine receptors in the sympathetic nervous system (Schmeller et al., 1994; Pothier et al., 1998; Australia New Zealand Food Authority, 2001).
Plant breeding programs developed to ensure the safety and wider utilisation of lupins lead to the development of ‘sweet’ lupins that exhibit non-toxic alkaloid levels (less than 150 mg.kg\(^{-1}\)) (Australia New Zealand Food Authority, 2001). Sweet lupin species include \textit{L. albus}, \textit{L. angustifolius}, \textit{L. mutabilis} (Andean lupin) and \textit{L. luteus} (yellow lupin). These species display a wide variety of applications and are cultivated as either green or organic manure, an animal stockfeed or for human consumption (Erbas \textit{et al.}, 2005).

As a green manure, lupins are an organic and sustainable means of improving soil quality and nitrogen levels, by facilitating the conversion of atmospheric nitrogen to ammonia (Cazzato \textit{et al.}, 2012). This application is possible due to the formation of root nodules in the 1 – 2 metre tap root system of lupins, which facilitate symbiotic interactions of the plant with nitrogen-fixing rhizobia such as \textit{Bradyrhizobium lupini} (Kurlovich \textit{et al.}, 2002; Shulze \textit{et al.}, 2006; Faest, 2010). In Australia, lupins are grown in rotation with cereal crops such as wheat (\textit{Triticum aestivum}) as an effective and economic means of broad-acre nitrogen distribution (Evans \textit{et al.}, 1987).

The primary fate of lupins is the use of lupin seeds for animal consumption, either as a dehulled kernel meal or whole grain. Cattle and poultry industries are the biggest consumers of lupins while aquaculture has recently emerged as a minor consumer, utilising lupin meal in the diets of salmon and prawn (Greirson \textit{et al.}, 1991; Australian Bureau of Agricultural and Resource Economics, 2007; Glencross, 2008). The integration of lupins into livestock industries is attributed to grain composition, as lupins express comparably high protein content with fewer anti-nutritional factors and starches than legumes such as soybean. Lupins may also be grown in a wider range of climates and soil types than soybean, making them an attractive alternative dietary commodity (Martínez-Villaluenga \textit{et al.}, 2006; Cazzato \textit{et al.}, 2012).

Human consumption of lupin seeds is also increasingly prevalent. Although dehulled seeds were traditionally consumed whole, modern lupin consumption consists primarily of lupin flour (finely ground kernel) in pasta, tofu, bread, beverages and imitation meat products marketed as an alternative protein source with various nutritional benefits (Erbas \textit{et al.}, 2005; Resta \textit{et al.}, 2008).
1.1.3 Nutritional Value and Health Benefits of Lupins

As lupin seeds have become increasingly relevant to animal diets, the nutritional value of seeds has been extensively investigated (Lampart-Szczapa et al., 2003; Bhardwaj et al., 2004; Erbas et al., 2005; Sujak et al., 2006; Muzquiz et al., 2011). Table 1.1 illustrates the typical gross compositions of three dietary lupin species.

Table 1.1: Typical gross composition (%) of the dietary lupin species L. angustifolius, L. albus and L. luteus (modified from Glencross, 2008).

<table>
<thead>
<tr>
<th>Lupin Component</th>
<th>L. angustifolius</th>
<th>L. albus</th>
<th>L. luteus</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Seed</td>
<td>Kernel</td>
<td>Seed</td>
</tr>
<tr>
<td>Hull</td>
<td>24</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>Moisture</td>
<td>9</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Protein</td>
<td>32</td>
<td>41</td>
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<td>Polysaccharides</td>
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</tr>
<tr>
<td>Oligosaccharides</td>
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<td>7</td>
</tr>
<tr>
<td>Minor Components</td>
<td>3</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

As exhibited by Table 1.1, lupin seeds are high in protein (30 – 40% dry weight in whole seed with interspecies variation due to environmental and genetic factors) and are therefore a valuable alternative source of dietary protein (Greirson et al., 1991; Erbas et al., 2005). Seeds also display similar percentages of available dietary fibre, particularly non-starch polysaccharides (cellulose and hemicellulose), which compose the majority of the outer seed hull (Evans et al., 1993; Lampart-Szczapa et al., 2006; Martínez-Villaluenga et al., 2006). Lupin seeds also possess high levels of essential amino acids, particularly leucine (3.2% dry matter in L. angustifolius seed), threonine, lysine and phenylalanine (1.8% dry matter in L. angustifolius seed) and nutritionally valuable levels of thiamine (B₁), riboflavin (B₂) and niacin (B₃) (Torres et al., 2005; Glencross, 2008). Relative to other legumes, lupins also display comparably low levels of anti-nutritional compounds including trypsin inhibitors, phytate, saponins and lectins (Erbas et al., 2005; Torres et al., 2005; Sujak et al., 2006; Sweetingham and Kingwell, 2008).

A number of health benefits have also been proposed from the inclusion of lupins in the human diet. For example, the enrichment of white bread with lupin kernel flour reduced total food intake.
by 30%, reduced food intake at a subsequent meal by 15% and significantly increased self-reported satiety in a controlled, randomised study over 4 weeks with 16 individuals. Although the small sample size necessitates a larger study in the future, it was suggested that the inclusion of lupins in the diet might therefore reduce food intake and obesity (Lee et al., 2006). Fontanari et al. (2012) reported significant reductions of total plasma cholesterol levels in hamsters (n = 8) with induced hypercholesterolaemia after receiving a diet rich in saturated fatty acids supplemented with whole lupin seeds or lupin protein isolates for 4 weeks, suggesting lupins may aid in reducing blood lipids and therefore risk of cardiovascular disease and atherosclerosis. Reviews by Hodgson and Lee (2008) and Lee et al. (2008) have also suggested various benefits to meal enrichment with lupins including reduced blood pressure, reduced risk of cardiovascular disease and improved glycemic control due to the high protein and fibre content of lupin seeds.

### 1.1.4 Pharmacologically Active Minor Components of Lupins

Several reports have recently identified various pharmacologically active phytochemicals in lupins. For example, Ranilla et al. (2009) identified the antioxidant isoflavone genistein in the hull and cotyledons of *L. mutabilis* via liquid chromatography (LC) analysis of a methanol seed extract. Siger et al. (2012) similarly detected two apigenin derivatives with antioxidant properties in methanol extracts of *L. albus*, *L. luteus* and *L. angustifolius* seeds via Liquid Chromatography – Mass Spectrometry (LC-MS). Torres et al. (2005) utilised LC analysis of an ethanol extract of *L. angustifolius* to identify the antioxidant vitamin E isomers α-, β-, γ- and δ-tocopherol. Hamama and Bhardway (2004) identified anti-tumour phytosterols in *L. albus* including stigmasterol and campesterol, as well as the anti-inflammatory phytosterol β-sitosterol (Loizou et al., 2009), via thin layer chromatography (TLC) and GC analysis. In addition, Hamama and Bhardway (2004) determined the pharmacologically active phytosterol lupeol was a minor constituent of the lipid fraction of *L. albus* seeds. However, there is limited data on the occurrence of lupeol in other lupin species and to the knowledge of the author, no quantitative data of the abundance of lupeol in lupin seeds.
1.2 Lupeol

Lupeol is a pentacyclic triterpenoid alcohol and secondary metabolite biosynthesised in the cytosol of eukaryotic cells from the steroid precursor squalene (Gallo and Sarachine, 2009). Lupeol displays an exact mass of 426.386166 g, chemical formula of C_{30}H_{50}O and a chemical structure as illustrated in Figure 1.2 (Breitmaier, 2006; Wal et al., 2010).

![Chemical structure of lupeol](image)

**Figure 1.2:** Chemical structure of lupeol (retrieved from Siddique and Saleem, 2011).

Lupeol has been broadly identified in the plant kingdom and is notably abundant in dietary fruits and vegetables such as mango (Prasad et al., 2008a), strawberry, grapes (Saleem, 2009), tomato (Bauer et al., 2004) and cabbage (Martelanc et al., 2007), nuts such as hazelnut (Azadmard-Damirchi, 2010) and medicinal plants including ginseng (Beveridge et al., 2002) and Aloe vera (Lawrence et al., 2009). Lupeol has also been identified in plant species used in traditional and folk medicines including *Trichodesma amplexicaule*, a plant used in Indian folk medicine to treat dysentery (Singh, 2007), *Strobilanthes cusia*, a tropical species used in traditional Chinese medicine to treat influenza, viral pneumonia, mumps and severe acute respiratory syndrome (Tanaka et al., 2004) and *Pimenta racemosa*, an invasive myrtle used in the Dominican Republic to treat rheumatism (Fernández et al., 2001). However, lupeol has also been detected in the cup sponge *Axinella infundibuliformis* (Lutta et al., 2008) and the medicinal mushroom *Inonotus obliquus* (Kahlos, 1989), suggesting the compound occurs in multiple eukaryotic kingdoms.
Although there is currently limited data on the bioavailability of lupeol in vivo, lupeol has been identified as non-toxic in animal models in acute doses up to 2000 mg kg\(^{-1}\) body weight (Bani et al., 2006; Lee et al., 2007c; Saleem et al., 2008), indicating the compound may be a safe dietary component. Lupeol has also received much attention from the scientific community by displaying a spectrum of beneficial pharmacological activities as both a therapeutic and preventative agent (reviewed by Gallo and Sarachine, 2009; Saleem, 2009; Siddique and Saleem, 2011).

1.2.1 Pharmacological Activities of Lupeol

The biological activities of lupeol have been broadly investigated in research encompassing therapeutic and prophylactic applications using in vitro and in vivo approaches. Existing data suggests lupeol displays varying levels of efficacy against a multitude of pathogens, diseases and potentially harmful endogenous biochemical processes (reviewed by Gallo and Sarachine, 2009; Saleem, 2009; Siddique and Saleem, 2011), some of which are summarised in Figure 1.3.

**Figure 1.3:** Pathogenic microorganism activities, diseases and destructive biochemical processes prevented or alleviated by lupeol (modified from Siddique and Saleem, 2011).

Figure 1.3 illustrates that lupeol exerts antibacterial activities, reported against Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (both associated with
respiratory infection) and Gram-positive bacteria such as *Enterococcus faecalis*, a member of the human intestinal flora responsible for nosocomial bloodstream and urinary tract infections (Ahamed *et al.*, 2007; Lutta *et al.*, 2008; Shai *et al.*, 2008). For example, Ahamed *et al.* (2007) extracted lupeol from the Indian medicinal tree *Grewia tiliaefolia* (used to treat pneumonia and bronchitis). By inoculating molten nutrient agar in Petri plates with 100 µL of nutrient broth inoculum (108 CFU.mL⁻¹), application of lupeol (1 mg.mL⁻¹ dissolved in 10% aqueous dimethyl sulfoxide) for 24 hours resulted in a mean inhibitory zone (MIZ) of 24.1 mm against 9 strains of *P. aeruginosa*. The inhibition of lupeol against *P. aeruginosa* was greater than the standard antibacterial agent ciprofloxacin (1 mg.mL⁻¹ in distilled water), a topoisomerase inhibitor, which induced a MIZ of 22.7 mm against the same 9 strains. Lupeol also induced a MIZ of 18.1 mm against 9 strains of *K. pneumonia*. However, lupeol was not as effective as the standard against *K. pneumonia*, which exerted a MIZ of 18.9 mm.

Figure 1.3 also indicates lupeol displays antiviral activities. For example, Tolo *et al.* (2010) investigated the activity of lupeol isolated from the Kenyan medicinal plant *Carissa edulis* against Herpes Simplex Virus Type I (HSV-1). Using confluent Vero E6 cells infected with HSV-1 (at a multiplicity of infection ratio of 5), cultures treated with 0 - 10 µg.mL⁻¹ lupeol for 24 hours displayed a dose-dependant reduction in cell viral yield with a maximum reduction of 98.3% plaque forming units per mL at 10 µg.mL⁻¹ (determined by serial dilution of cell supernatants with plaque inhibition assays). Lupeol has therefore demonstrated antimicrobial activity comparable to that of standards and may therefore be an effective dietary antimicrobial agent.

Lupeol has also demonstrated activities against locally induced inflammation (Figure 1.3). For example, Vasconcelos *et al.* (2008) conducted a comparative study of lupeol and the clinical glucocorticoid anti-inflammatory agent dexamethasone (DXM). Prophylactic oral treatment of 60 mg.kg⁻¹ body weight of lupeol to mice (n = 7) 2 hours prior to induced inflammation (aerosolised 1% ovalbumin in saline) limited airway infiltration of inflammatory cells (lymphocytes, macrophages and eosinophils) to approximately 3 fold less than that in mice receiving no therapeutic agent (n = 7). Although the result was not statistically different to mice receiving
30 mg.kg$^{-1}$ DXM (n = 7), lupeol afforded comparable anti-inflammatory activity and may be a useful naturally occurring prophylactic against inflammatory conditions.

Lupeol has also demonstrated in vitro and in vivo activities against the initiation, promotion, proliferation, invasiveness, metastasis and growth of various cancers, including prostate, skin, pancreatic, oral and blood cancers (Aratanechemuge et al., 2004; Saleem et al. 2005a; Lee et al. 2007c; Prasad et al., 2008b; Saleem et al., 2008, Nigam et al., 2009; Siddique et al., 2011). For example, Lee et al. (2007c) examined the in vivo effect of lupeol against tumours generated from metastatic oral squamous carcinoma cell lines in mice. Using a charge-coupled device camera to visualise tumour cells (transfected with a luciferase gene that induced bioluminescence upon intraperitoneal (IP) administration of 3 mg luciferin), mice receiving IP administration of lupeol at 2 mg for 30 days displayed an 85% reduced tumour volume relative to untreated animals. Lupeol was also more effective than the clinical chemotherapy drug cisplatin, which induces DNA crosslinking and subsequent apoptosis (Fuertes et al., 2003). IP administration of cisplatin at 5 mg.kg$^{-1}$ body weight for the same time period resulted in a 43% tumour volume reduction relative to untreated controls. Lupeol (5 - 20 μM) also reduced cancer cell invasiveness and metastasis, visibly slowing the rate of cell migration and wound closure compared to untreated controls when confluent monolayers of cultured oral carcinoma cells (5 x 10$^4$ cells per plate) were physically scraped with a pipette to generate an acellular area across the plate. Further, lupeol (22.2 μM) visibly altered cell morphology and increased expression of epithelial protein markers relative to controls when applied to confluent cultures, indicating transdifferentiation from mesenchymal to epithelial phenotypes, reversing the development of invasive tumours derived from epithelial cells. Confluent cultures of cells derived from tongue and metastatic oral squamous cell carcinomas were also treated with 100 μM lupeol and incubated for 48 hours, resulting in 100% growth inhibition of both cell lines relative to untreated controls.

Further reviews of the bioactivities activities of lupeol against diseases and pathogens illustrated in Figure 1.3 have been published by Gallo and Sarachine (2009), Saleem (2009), Siddique and Saleem (2011) and Wal et al. (2011).
1.2.1.1 Bioprotective Activities of Lupeol

In addition to its variegated effects on eukaryotic and prokarytic biology, lupeol exhibits specific bioprotective activities (Vidya et al., 2002; Sudhaharsan et al., 2005; Nigam et al., 2007; Kumari and Kakkar, 2012). For example, lupeol administered orally to rats (n = 5) at 150 mg.kg\(^{-1}\) body weight for 30 consecutive days, demonstrated hepatoprotective activities against a subsequent insult by acetaminophen, an analgesic and antipyretic drug inducing cellular oxidative stress. Hepatoprotection was measured by lupeol minimising induced changes in histopathological status and preventing the resulting production of reactive oxygen species and increases in lipid peroxidation (Kumari and Kakkar, 2012). Lupeol has also displayed nephroprotective attributes, limiting tubular damage and the excretion of stone-forming constituents in rats with induced urolithiases fed 35 mg.kg\(^{-1}\) body weight of lupeol (Vidya et al., 2002). Sudhaharsan et al. (2005) observed declines in several enzymatic antioxidants (superoxide dismutase, glutathione peroxidase and catalase) and non-enzymatic antioxidants (GSH and vitamins C and E), as well as enzymatic perturbations in lactate dehydrogenase, in the cardiac tissue of rats (n = 6) receiving IP administration of the chemotherapeutic, DNA alkylating agent cyclophosphamide. These responses, however, did not occur in rats fed 50 mg.kg\(^{-1}\) body weight lupeol (n = 6) 10 consecutive days prior to administration of the drug, suggesting lupeol induced a cardioprotective effect. Further protective activities have been explored by Nigam et al. (2007), reporting topical application of 200 µg of lupeol to murine skin (n = 20) 1 hour after topical application of the laboratory carcinogen 7,12-dimethylbenz(a)anthracene, inhibited induced DNA strand breakages by 43%, indicating potential chemoprotective properties. However, despite various demonstrations of protective and therapeutic activities of lupeol, neuroprotective properties do not appear to have been previously explored, indicating a knowledge gap in the spectrum of bioactivities demonstrated by lupeol.

1.3 Gas Chromatography – Mass Spectrometry

Gas Chromatography – Mass Spectrometry (GC-MS) is an hybrid instrumental analysis platform comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS) for the sequential
separation, detection and identification of a broad range of volatile and thermally labile compounds (McMaster, 2008; Sparkman et al., 2011). As a powerful, sensitive and selective technique, GC-MS is advantageous for the analysis of compounds in complex matrices and the acquisition of qualitative and quantitative data, detecting analytes at quantities as low as femtomolar amounts (Hübschmann, 2001; Sparkman et al., 2011). Such capabilities are possible because of the synergistic operation of the GC, which separates and analyses components of a sample by mobile and stationary phase interactions, with that of the MS, which detects analytes eluting from the GC, generates ionisation products and resolves these ions for compound identification (Niessen, 2001).

1.3.1 Gas Chromatography Instrumentation

Gas Chromatography is an analytical separation technique employed for the determination of analytes via selective distribution between a gaseous mobile phase and liquid or solid stationary phase (Merves and Goldberger, 2010). Instrumentation consists of an injector, column and detector. Initially, precise quantities of a sample are introduced to the GC via a manually or mechanically-operated syringe, entering a temperature-controlled injector where samples are volatilised to the gas phase for dissolution in a mobile phase of a high purity, inert carrier gas (typically helium, hydrogen or nitrogen), which transmits the sample through the instrument (Hübschmann, 2001; McMaster, 2008). Analytes are then directed into a coiled capillary or packed column composed of fused silica or glass housed within a temperature-controlled oven (Skoog et al., 2007). Separation of the sample is achieved within the column through differential interactions of analytes in the mobile phase, with the stationary phase, which lines the internal surface of the column. Different analytes ideally exhibit variable sorption/desorption events between phases, resulting in differential retention within the column and varied rates of column migration. The desired result is the elution of individual analytes at unique times to ensure compound isolation and effective analysis (Niessen, 2001; Skoog et al., 2007). A detector records the quantity of each eluting compound and generates corresponding signals, which are processed by a data integrator (McNair and Miller, 2009). A chromatogram composing signal intensities
(corresponding to amounts of compound detected), as a function of respective time of elution (retention time or RT) is presented as the data output (Hübschmann, 2001; Skoog et al., 2007). In GC-MS, an interface and transfer line then transmits the sample to the MS, whilst simultaneously removing the carrier gas (Yuwono and Indrayanto, 2005).

### 1.3.2 Mass Spectrometry Instrumentation

Mass Spectrometry is principally concerned with the production, separation and detection of gas-phase ions (Niessen, 2001). Instrumentation consists of an ion source, mass analyser and ion detector, each of which is maintained under vacuum to prevent undesired ion collisions (Downard, 2004). In the MS, a gas-phase sample enters through an inlet and is immediately ionised at the ion source (Skoog et al., 2007). Multiple ionisation techniques are possible including chemical ionisation, which utilises reagent gaseous ions, and field ionisation, which uses a high-potential electrode to generate ions from analyte molecules (Niessen, 2001; Skoog et al., 2007). The simplest and most common ionisation technique is electron ionisation (EI), where gaseous analytes are bombarded with an electron beam, typically at a voltage of -70 eV (McNair and Miller, 2009). Analytes collide with electrons, primarily generating cations, where $M^+$ designates the molecular ion, possessing the same mass as the molecular weight of the analyte (Downard, 2004). Molecules also fragment as a result of the imparted energy from collisions, generating variable cleavage products of masses less than $M^+$ (Downard, 2004, Niessen, 2001). Under constant conditions, molecules fragment reproducibly, generating precise patterns and proportions of particular ions. This reproducible fragmentation is central to the qualitative and quantitative capabilities of the instrument (McMaster, 2008). A series of charged accelerators and a repeller within the ion source then propel ions into the mass analyser (Skoog et al., 2007).

In the mass analyser, ions are separated by differing mass-to-charge ratio ($m/z$). As generated ions typically possess a single positive charge, $m/z$ values generally equal the mass of the molecule or fragment formed (Barker, 1999). A number of analysers are commercially available for separation in time or space, in response to electric and/or magnetic fields (Watson and Sparkman, 2007). A common mass analyser is the quadrupole mass filter (QMF). Composed of two pairs of
cylindrical, parallel rods, the QMF generates variable electrical fields to selectively passage ions by applying voltages of opposite charge to adjacent rods in the form of direct current (DC) and an oscillating radiofrequency (RF) (Downard, 2004; Skoog et al., 2007; Sparkman et al., 2011). Quadrupole rods operate antagonistically to stabilize ions of a desired m/z, allowing them to traverse the quadrupole in a corkscrew, three-dimensional sine wave formation and reach the ion detector (illustrated in Figure 1.4), while other ions collide with the quadrupole surface and do not reach the detector (Barker, 1999; Skoog et al., 2007; McMaster, 2008).

Figure 1.4: Typical QMF illustrating ion path (left to right). Ions enter the QMF through the ion source and traverse across Z, Y and X-axes between opposing pairs of parallel rods towards the detector (retrieved from Sparkman et al., 2011).

The ion detector then converts the ion beam to an electrical signal, which is transmitted to a signal processor responsible for data output as mass spectra, illustrating m/z ratios of ions with relative abundances (Niessen, 2001).

QMFs are capable of analysing ions with a m/z up to 4000 and depending upon the mode of operation, may monitor specific ions with a single m/z (achieved in ‘Fixed’ mode) or passage a range of ions with varied m/z by continuously altering the applied RF/DC ratio (achieved in a ‘Scan’ mode) (McNair, 2009). The QMF exhibits several advantages over other mass analysers including lower production costs, faster scan capacity, more compact and lightweight design and a more robust operation (Barker, 1999; Downard, 2004; Skoog et al., 2007).
A prominent alternative to the QMF is the time-of-flight (TOF) mass analyser, which generates ions with consistent kinetic energies and accelerates them into a flight tube, where ions are spatially separated. As ion mass is inversely proportional to velocity, heavier ions (with larger \( m/z \)) travel more slowly and reach the detector at the end of the flight tube at later times, relative to ions with lower \( m/z \), which traverse the flight tube more rapidly (Skoog et al., 2007). The TOF is faster and more sensitive than the QMF but is generally more expensive (McNair, 2009).

Despite the capabilities of MS, structural information of analytes can be difficult to acquire with the generation of molecular ions that fail to fragment (Downard, 2004; Watson and Sparkman, 2007). Challenges may also arise with compounds coeluting from the GC, convoluting mass spectral output and complicating data analysis (Hübschmann, 2001). Such complications may be overcome with the implementation of multiple mass analysers in tandem mass spectroscopy (MS/MS). This is particularly useful in analyses requiring greater instrumental sensitivity or where minimal structural information is available on compound of interest. The use of MS/MS with a GC for analyte separation is therefore particularly relevant to trace analysis and compound detection in complex matrices (Hübschmann, 2001; Hsu and Drinkwater, 2001).

A prominent MS/MS technique is the triple quadrupole (QQQ) system, which employs three QMFs in series to generate additional ion fragmentation and analysis. QQQs function by quadrupole one (Q₁) performing ion analysis through the previously described mechanism of QMF operation. Ions are then transmitted to quadrupole two (Q₂), which operates as a collision cell in RF mode only to induce further ion decomposition through collisionally activated dissociation with a collision gas (such as \( \text{N}_2 \)). Q₂ then transmits ions of all \( m/z \) values to quadrupole three (Q₃) for supplementary mass analysis (Downard, 2004; Skoog et al., 2007; Watson and Sparkman, 2007).

The QQQ has many advantages over other systems including high sample throughput, improved sensitivity, simpler operation, modifiable quadrupole voltages and collision gases and can be used to perform a number of different scan types, which permit a wider range of applications (Watson
and Sparkman, 2007). Table 1.2 summarises several types of scan achieved by the QQQ, which can be variably applied depending upon the analytical goal and the sample analysed.

**Table 1.2:** Q₁ and Q₃ settings for GC-MS/MS QQQ analysis modes where ‘Scan’ indicates analysis of all m/z in a given range and ‘Fixed’ indicates a single m/z (modified from Hopfgartner, 2011).

<table>
<thead>
<tr>
<th>Scan Type</th>
<th>Q₁ Mode</th>
<th>Q₃ Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Scan</td>
<td>Scan</td>
<td>RF Only</td>
</tr>
<tr>
<td>Single Ion Monitoring</td>
<td>Fixed (single m/z)</td>
<td>RF Only</td>
</tr>
<tr>
<td>Product Ion Scan</td>
<td>Fixed (single m/z)</td>
<td>Scan</td>
</tr>
<tr>
<td>Precursor Ion Scan</td>
<td>Scan</td>
<td>Fixed (single m/z)</td>
</tr>
<tr>
<td>Selected Reaction Monitoring</td>
<td>Fixed (single m/z)</td>
<td>Fixed (single m/z)</td>
</tr>
<tr>
<td>Multiple Reaction Monitoring</td>
<td>Fixed (multiple m/z)</td>
<td>Fixed (multiple m/z)</td>
</tr>
</tbody>
</table>

Alternative MS/MS techniques include the use of a single QMF with a TOF mass analyser (Q-TOF), which has seen application in proteomics and offers significant mass accuracy and resolving power. Alternatively, the use of duel TOF mass analysers (in an analogous manner to dual QMFs in a QQQ system) is also useful in the identification of unknown compounds and affords rapid data generation and high-throughput analysis (Watson and Sparkman, 2007).

### 1.4 Applications of GC-MS

Due to the advantages in sensitivity, selectivity, robustness, separation power and capabilities in both qualitative and quantitative determinations, GC-MS has been applied to a variety of scientific fields including forensics, toxicology, pharmacology and environmental sciences (Hübschmann, 2001; McMaster, 2008; Sparkman et al., 2011). Additionally, GC-MS is a prominent instrumental technique in the emerging field of metabolomics (Kanani et al., 2008), while the field of residue analysis depends greatly upon the low limits of detection, selectivity and specificity afforded by GC-MS (De Brabander et al., 2011).

#### 1.4.1 Metabolomics

Metabolomics is the study of metabolites in biological samples under a given set of conditions, where metabolites are considered small molecules with a m/z of less than 1000 (De Hoffman and
Stroobant, 2007; Nielsen, 2007). The field is analogous to genomics, transcriptomics or proteomics as a systems biology approach to the analysis of the metabolome (Højar-Pederson et al., 2007). The metabolome is the complete set of metabolites involved in a cell, tissue or organism’s metabolic pathways and therefore concerns endogenous and exogenous components including peptides, vitamins, amino acids, sterols, proteins, fatty acids and other organic molecules, the number of which varies greatly between taxonomic groups (Fiehn, 2002; Wishart, 2008a; Jones et al., 2011). Metabolomics is a useful investigative approach in many fields due to high-throughput capabilities and lower costs per sample relative to proteomics or transcriptomics (Dunn, 2008; Jones et al., 2011). Metabolomics is also an holistic analytical tool, investigating final products of gene expression and providing phenotypic information (Dunn, 2008). Metabolomics can therefore be utilised to inform the physiological state of a biological object and is valuable in measuring gene function, response to environmental stimuli and biochemical responses to diseased states (Højar-Pederson et al., 2007; Ritsner and Gottesman, 2009).

Metabolomics encompasses a number of analytical strategies that may be variably employed depending upon the availability of resources and the nature of metabolite information required. Table 1.3 presents some common analytical approaches of metabolomics, which inform either the endometabolome (intracellular metabolites) or exometabolome (extracellular metabolites) with varying levels of quantitative information (Højar-Pederson et al., 2007; Dunn, 2008).

**Table 1.3:** Analytical strategies and associated terminology commonly employed in metabolomics investigations (modified from Shulaev, 2006; Højar-Pederson et al., 2007; Dunn, 2008).

<table>
<thead>
<tr>
<th>Strategy of Analysis</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untargeted Metabolite</td>
<td>Detection of numerous metabolites employing single or multiple analytical platforms to maximise coverage of the metabolome with identification and quantification where possible.</td>
</tr>
<tr>
<td>Profiling</td>
<td></td>
</tr>
<tr>
<td>Targeted Metabolite</td>
<td>Absolute quantification and identification of a single or small highly related set of metabolites after significant sample preparation.</td>
</tr>
<tr>
<td>Profiling</td>
<td></td>
</tr>
<tr>
<td>Metabolic Fingerprinting</td>
<td>High-throughput analysis providing a metabolic snapshot of intracellular metabolites, involving limited sample preparation, identification or quantification.</td>
</tr>
<tr>
<td>Metabolic Footprinting</td>
<td>High-throughput analysis providing a metabolic snapshot of extracellular metabolites with minimal identification or quantification with no metabolite extraction required.</td>
</tr>
</tbody>
</table>
1.4.2.1 Sample Preparation in Metabolomics

Metabolomics may potentially be performed on any biological object including cell cultures, bacteria and whole organisms (Jones et al., 2011). However, methods of sample preparation for analysis differ significantly depending upon the type of sample and the analytical goal. Sample preparation is therefore a crucial aspect of metabolomics but is typically conducted stepwise with harvesting, quenching, metabolite extraction and derivatisation, prior to instrumental analysis (Villas-Bôas, 2007).

Harvesting is the isolation of a sample for analysis, occurring simultaneously or immediately prior to the cessation of metabolic activity (quenching). In cell cultures, harvesting may be achieved by gentle cell scraping, the application of trypsin and ethylenediaminetetraacetic acid (EDTA) solutions, the addition of buffers such as isotonic phosphate-buffered saline (PBS) or with combinations thereof (Dettmer et al., 2011). Quenching is achieved through sudden changes in pH and/or temperature and is vital to ensure representative metabolite samples are obtained by inhibiting further metabolic activity from occurring during sample preparation. Quenching is further imperative due to the rapid turnover rates of metabolites, which may be less than 1 mM per second (Villas-Bôas, 2007; Álvarez-Sánchez et al., 2010).

Extraction concerns the degradation of cellular structures (cell lysis) and liberation of intracellular metabolites. The effectiveness of metabolomics analyses is particularly dependent upon extraction, ideally achieving efficient metabolite release from the cell, removal of contaminants such as proteins and salts, concentration of metabolites in the solvent and the extraction of metabolites into a solvent applicable for downstream instrumental analysis (Rodrigues et al., 2008; Álvarez-Sánchez et al., 2010). For these reasons, solvents such as methanol and/or water have proved effective in metabolomics sample preparation (Dettmer et al., 2011).

Derivatisation is a chemical modification procedure that increases the volatility of analytes otherwise not sufficiently volatile or thermally stable to reach the gas phase in the GC injector during GC-MS analysis (Niessen, 2001; Smedsgaard, 2007). Derivatisation is achieved by the application of reactants that reduce analyte polarity through functional group substitution.
Silylation (substitution of hydrogen with silicon-based silyl group) using reagents such as MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) is most widely used (Villas-Bôas et al., 2007; Watson and Sparkman, 2007).

1.4.2.2 Instrumental Analysis in Metabolomics

As a comparatively new field of post-genome biological research, metabolomics capabilities have been rapidly advanced by technological developments in molecular separation and identification (Wishart, 2008a). However, as metabolites display a broad spectrum of physical properties and structures, no single platform provides a complete metabolome analysis (Jones et al., 2011). A number of alternative platforms to GC-MS have therefore seen application in metabolomics.

For example, LC-MS utilises a liquid mobile phase (rather than the inert gas in GC-MS) to separate analytes through differential interactions with a sorbent (stationary phase) within a packed or capillary column. Similarly to GC-MS, the velocity of analytes moving through the column is a function of physical interactions occurring with the stationary phase. However, in LC-MS, the mobile phase is more integral to the success of the chromatographic separation and must contribute precisely to the balance of intermolecular forces between analyte and stationary phase (Skoog et al., 2007). LC-MS also uses distinctive methods of ionisation such as electrospray ionisation, whichemploy electrical energy to transfer ions from charged solvent droplets into a mass analyser such as a QMF or TOF (Ho et al., 2003).

Another alternative platform is Nuclear Magnetic Resonance spectroscopy (NMR), which measures the absorption of electromagnetic radiation of atomic nuclei by exposing analytes to intense magnetic fields, instead of the electron bombardment utilised in typical MS analyses (Skoog et al., 2007). This approach is particularly useful in the generation of structural information of compounds and was originally the preferred platform of analysis in establishing the field of metabolomics (Wishart, 2008b).

GC-MS, LC-MS and NMR therefore offer varying approaches to metabolomics, each of which continue to be utilised in the field today. Table 1.4 presents several advantages and disadvantages
related to the use of either platform, all of which must be considered in a decision of which instrument to utilise in a metabolomics analysis.

Table 1.4: Advantages and disadvantages of instrumental platforms commonly utilised in metabolomics (modified from Wishart, 2008a; Wishart, 2008b).

<table>
<thead>
<tr>
<th>Platform</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-MS</td>
<td>• Relatively inexpensive</td>
<td>• Sample not recoverable</td>
</tr>
<tr>
<td></td>
<td>• Good sensitivity (limit of detection in nM range)</td>
<td>• Requires sample derivitisation</td>
</tr>
<tr>
<td></td>
<td>• Large databases for metabolite identification</td>
<td>• Slow (20-30 minutes per sample)</td>
</tr>
<tr>
<td></td>
<td>• Detects most organic and some inorganic molecules</td>
<td>• Requires sample separation</td>
</tr>
<tr>
<td></td>
<td>• Good separation reproducibility</td>
<td>• Thermally unstable molecules not applicable</td>
</tr>
<tr>
<td></td>
<td>• Less ion suppression in co-eluting compounds (than LC-MS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Modest sample size requirements (&lt; 50 µL)</td>
<td></td>
</tr>
<tr>
<td>LC-MS</td>
<td>• No sample derivitisation required</td>
<td>• Relatively expensive</td>
</tr>
<tr>
<td></td>
<td>• Multiple modes of separation</td>
<td>• Sample not recoverable</td>
</tr>
<tr>
<td></td>
<td>• Sample volatility not required</td>
<td>• Slow (20-30 minutes per sample)</td>
</tr>
<tr>
<td></td>
<td>• Detects most organic and some inorganic molecules</td>
<td>• Limited databases for metabolite identification</td>
</tr>
<tr>
<td></td>
<td>• Minimal sample size requirements (&lt; 10 µL)</td>
<td>• Susceptible to ion suppression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Less reproducible RTs than GC-MS</td>
</tr>
<tr>
<td>NMR</td>
<td>• Fast (2-3 minutes per sample)</td>
<td>• Relatively expensive</td>
</tr>
<tr>
<td></td>
<td>• No sample derivitisation required</td>
<td>• Not very sensitive (limit of detection in µM range)</td>
</tr>
<tr>
<td></td>
<td>• No sample separation required</td>
<td>• Cannot detect salts, inorganic ions or non-protonated compounds</td>
</tr>
<tr>
<td></td>
<td>• Detects all organic classes</td>
<td>• Requires large sample sizes (&gt; 500 µL)</td>
</tr>
<tr>
<td></td>
<td>• Non-destructive to sample</td>
<td></td>
</tr>
</tbody>
</table>

1.4.2.3 Data Analysis in Metabolomics

As a systems biology approach, metabolomics analyses generate vast quantities of data requiring specialised statistical and bioinformatics tools to derive biologically significant information (Shulaev, 2006). Principal Component Analysis (PCA) is the most widely utilised statistical technique for the analysis of metabolomics data (Gibney et al., 2008). PCA is utilised to explore overall variation of a sample set by spatially associating samples with similar metabolic profiles and therefore distancing samples of dissimilar profiles, to allow for the detection of outliers.
(Affolter et al., 2009). PCA effectively reduces a large number of variables (metabolite peak areas detected by the instrument) into a small number of principal (statistical) components (PCs), which explain the variability observed between all samples (Affolter et al., 2009; Xu and Schaefer, 2012). The output of PCA analysis is a ‘scores’ plot, which clusters similar metabolic profiles together across two orthogonal PCs, with a ‘loadings’ plot, which displays the influence of variables (metabolites) upon the variation observed in the scores plot (Vliet et al., 2008; Xu and Schaefer, 2012). A PC that best explains the variation in the sample set can then be selected to further evaluate variation between treatment groups, according to changes in metabolites most influencing the variance between samples (Affolter et al., 2009).

### 1.4.2 Residue Analysis

Residue analysis is a broad scientific field encompassing trace-level analysis of toxicologically and pharmacologically significant compounds. Instrumental platforms are typically utilised to maximise the sensitivity and specificity of analysis and increase the number and variety of compounds detected in multi-residue analyses. An economically significant area of residue analysis concerns the determination of pesticide residues - depositions of active components, metabolites and degradation products of pesticides (Winteringham, 1971; Alder et al., 2006; De Brabander et al., 2011). Pesticide residues present a global health concern, with many residues toxic to organisms other than those targeted in application and capable of penetrating terrestrial and aquatic food chains whilst remaining environmentally persistent (Winteringham, 1971; Alder et al., 2006).

Despite the capabilities of GC-MS, methodologies of sample preparation represent a bottleneck in instrumental analysis and significantly impact limits of detection and quantitative capabilities (De Brabander et al., 2011). Depending upon the analytical goal, a variety of methods are currently available for sample preparation of samples for pesticide residue detection (reviewed by Zhang et al., 2012). A notable procedure is the QuEChERS approach, a technique presenting unique advantages to residue determinations with various novel applications (Anastassiades et al., 2003; Wilkowska and Biziuk, 2011).
1.4.2.1 The QuEChERS Approach

QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) is a descriptive acronym for a multistep, micro-scale chemical extraction methodology developed for pesticide residue determinations in fruit and vegetables (Anastassiades et al., 2003). QuEChERS is a globally recognised technique in food analysis, having garnered popularity from both commercial and research-based scientific sectors (Romero-González et al., 2008; Wilkowska and Biziuk, 2011). This popularity is due to both the advantages described in the method’s namesake and the superiority of the technique over traditional pesticide residue sample preparation techniques, promising greater sample throughput (Romero-González et al., 2008), less solvent wastage and glassware requirements, reduced costs and labour (Wilkowska and Biziuk, 2011) and the application of samples to instrumental analysis techniques such as GC-MS and LC-MS (Lesueur et al., 2008; Maštovská et al., 2010).

QuEChERS is currently utilised by the Separation Sciences and Metabolomics Laboratory at Murdoch University for the determination of pesticide residues from a variety of commodities including wheat, barley, oats and lupins. Figure 1.5 illustrates an example of a QuEChERS extraction protocol employed at the Murdoch University laboratory.

**Figure 1.5:** Example of a QuEChERS protocol employed for the extraction of pesticide residues into an extraction solvent.
QuEChERS extraction protocols facilitate the removal of compounds of interest into a polar extractant solution, followed by salting-out and liquid-liquid partitioning, allowing compounds to be recovered in an organic phase (Anastassiades et al., 2003; Díez et al., 2006; Majors, 2007). As illustrated in Figure 5, the commodity of choice is first comminuted to maximise the substrate surface area exposed to the extraction solvent, thereby increasing extraction efficacy and generating an homogenous mixture of material (Anastassiades et al., 2003). Water is added to representative subsamples to penetrate grain pores, inducing swelling and allowing the commodity to become more accessible to the extraction solvent (Pinto et al., 2010). In Figure 5, acetonitrile is the extraction solvent of choice, although alternatives include acetone and ethyl acetate (Maštovská and Lehotay, 2004). However, acetonitrile is advantageous for multiresidue extraction as it can be utilised in downstream instrumental analysis (including GC-MS) and readily partitions from water with the addition of MgSO₄ and NaCl, thereby providing effective phase-separation of compounds of interest (Maštovská and Lehotay, 2004; Díez et al., 2006). The addition of acetic acid in acetonitrile has also demonstrated improved recoveries of pesticides including dicofol and chlorothalonil by adjusting solvent pH, thereby increasing the range of compounds extracted (Maštovská and Lehotay, 2004). Samples are repeatedly shaken throughout the procedure to agitate the matrix and facilitate compound recovery. Homogenisation is also utilised to further reduce particle size and increase extraction efficacy (Anastassiades et al., 2003).

QuEChERS extraction kits are commercially available mixtures of various chemical components packaged in precise quantities, which facilitate compound recovery, remove contaminants and prevent compound degradation. The kit of choice is often dependant on the sample volume being investigated (United Chemical Technologies, 2012). Extraction kits typically include MgSO₄ and NaCl, which modify the polarity of the extraction solvent to increase compound extraction, particularly in the recovery of polar analytes, thereby improving analyte recovery and improving partitioning of the solvent (Díez et al., 2006). MgSO₄ however, induces an exothermic rehydration reaction (reaching temperatures of 40 – 45°C), necessitating the use of ice to minimise thermal degradation of heat sensitive compounds, as employed in Figure 1.5.
Sodium citrate buffers are also included in extraction kits to stabilise matrix pH within a range that maximises compound recovery whilst minimising degradation, ionisation and co-extraction of contaminants (Anastassiades et al., 2003).

Centrifugation then separates the extraction solvent containing compounds of interest, from residual plant material, reagents and water. Sample freezing can also be performed to physically separate contaminants with poor solubility in the solvent, such as lipids, waxes and sugars (Anastassiades et al., 2007; Payá et al., 2007). Following freezing, a portion of the solvent is centrifuged to further remove poorly soluble contaminants, with an aliquot of extractant subjected to clean-up as outlined in Figure 1.6.

![Figure 1.6: Example of a QuEChERS clean-up protocol employed for the removal of sample contaminants and residual water prior to instrumental analysis.](image)

Figure 1.6 illustrates a QuEChERS clean-up protocol, which removes co-extracted matrix contaminants via dispersive solid-phase extraction (Mašťovská and Lehotay, 2004; Díez et al., 2006). Clean-up is achieved by the addition of a second QuEChERS kit containing C_{18}, primary secondary amine (PSA) sorbent and anhydrous MgSO_{4}, which remove long chain fatty acids, sterols, lipids, residual water other non-polar contaminants. Cunha et al. (2007) demonstrated a 64% reduction in co-extractives by weight via the addition of 50 mg PSA and 150 mg of anhydrous MgSO_{4} per mL of extract in the analysis of green olives. Cunha et al. (2007) further
demonstrated the importance of clean-up protocols to downstream instrumental analysis, as the presence of contaminants decreased background chromatographic responses, altered response of compounds of interest and decreased the efficiency of chromatographic separation.

QuEChERS is also a versatile methodology and has been applied to the determination and simultaneous quantification of over 100 pesticides (Lesueur et al., 2008). Further, QuEChERS has been successfully applied to a broad range of substrates including orange, grape, cucumber, red wine (Payá et al., 2007), onion, tomato (Lesueur et al., 2008), fruit juice (Romero-González et al., 2008), olive and olive oil (Garcia-Reyes et al., 2007), numerous cereal grains (Maštovská et al., 2010), fruit tree leaves (González-Curbelo et al., 2011), animal tissue (Rawn et al., 2010), with other substrates reviewed by Wilkowska and Biziuk (2011). Modified QuEChERS approaches have further expanded the scope of the technique, recovering other significant non-pesticide residues including veterinary drugs in animal tissue (Stubbings and Bigwood, 2009), antibiotics in human blood (Stevens, 2011), environmentally-persistent pharmaceuticals and their metabolites in soil (Bragança et al., 2012), antimicrobials in honey (Bargańska and Namieśnik, 2011; Hayashi et al., 2011) and acrylamide in human foodstuffs (Maštovská and Lehotay, 2006). However, the use of a QuEChERS approach for the recovery of lupeol from lupins has not been formally described.

1.5 Aims

As lupin seeds are commercially produced for consumption by livestock and humans, a thorough understanding of the chemical composition of these seeds is essential to ensure the health and safety of consumers. Although the phytochemical lupeol has been identified in lupin seeds, no quantitative data for the amount of lupeol present in is seeds currently available. There is also an absence of data on where within the seed structure lupeol may be expressed. A quantitative analysis of the compound in lupin seeds and seed fractions would therefore complement existing data on lupin-derived lupeol and better inform consumers on the implications of lupin seed consumption.
There is also no published data on whether lupeol exhibits neuroprotective properties, despite demonstrating several other pharmacological and bioprotective activities. An investigation of the ability of lupeol to protect neuronal cell against a neurotoxin would therefore be a valuable addition to existing knowledge in the field.

Despite the pesticide residue extraction technique QuEChERS demonstrating a broad range of applications, there are no published data on the use of QuEChERS to extract lupeol from lupins. The successful demonstration of this application would therefore identify a novel approach to simultaneously extract toxic and therapeutic compounds in a single analysis.

This project will therefore utilise a QuEChERS approach to extract lupeol from samples of lupin seeds and seed fractions and generate quantitative data on the compound using GC-MS. An established in vitro neuronal cell culture model will be employed to investigate the impact of lupeol treatment and activity of lupeol as a protective against a neurotoxic compound.

The aims of this work are therefore to:

i. Apply the QuEChERS approach to the extraction of lupeol from lupin seeds.

ii. Quantitatively determine the amount of lupeol in whole lupin seeds and seed fractions.

iii. Investigate the metabolic impact of lupeol on metabolite profiles of neuronal cells.

iv. Investigate the activity of lupeol as a potential neuroprotectant against a neurotoxic compound.
2. Materials and Methods

2.1 Materials

2.1.1 Reagents

Acetonitrile, ethanol, methanol, water and hexane were of LCMS grade and obtained from Fisher Scientific (New Jersey, USA). Formic acid (99% purity) and pyridine were purchased from Ajax FineChem (Taren Point, Australia). Mirex,acenaphthene-d10, chrysene-d12 and phenanthrene-d10 internal standards were purchased from Leco (Baulkham Hills, Australia). Lupeol (≥94% purity), acetic acid (≥99% purity), 13C-Sorbitol, PBS (Phosphate-Buffered Saline), MSTFA, n-alkanes (C_{10}, C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32}, C_{36}), methoxyamine hydrochloride, penicillin/streptomycin, Dulbecco’s modified Eagle’s medium (DMEM), trypsin and glutamine were purchased from Sigma-Aldrich (Castle Hill, Australia). Foetal calf serum was purchased from SAFC Biosciences (Brooklyn, Australia).

QuEChERS extraction and clean-up kits were purchased from United Chemical Technologies (Pennsylvania, USA). Extraction kits used for the extraction of lupin matrix from whole seeds and seed fractions except germ contained either 4 g anhydrous MgSO_4, 1 g NaCl, 1 g sodium citrate dehydrate and 0.5 g sodium citrate sesquihydrate supplied in a 50 mL centrifuge tube, or 6 g anhydrous MgSO_4, 1.5 g NaCl, 1.5 g sodium citrate dehydrate and 0.75 g sodium citrate sesquihydrate supplied in a mylar pouch. An extraction kit containing 2 g anhydrous MgSO_4, 0.5 g NaCl, 0.5 g sodium citrate dehydrate and 0.25 g sodium citrate sesquihydrate supplied in a 15 mL centrifuge tube was used for the extraction of an isolated lupin seed germ sample. Clean-up kits used for lupin matrix from whole seeds and seed fractions except germ contained 1200 mg anhydrous MgSO_4, 400 mg PSA and 400 mg C_{18} and were supplied in a mylar pouch. A clean-up kit containing 150 mg anhydrous MgSO_4, 50 mg PSA and 50 mg C_{18} was used for a lupin seed germ sample.

2.1.2 Biological Samples

Samples of organic *L. angustifolius* seeds and isolated seed fractions for lupeol quantification were supplied by the CBH Group (Australian Grains Centre Laboratory, Forrestfield, Australia). Isolated seed fractions consisted of whole seed, isolated kernel (cotyledon), isolated hull (seed
coat) and pure germ (embryo). An immortalised adherent B50 rat cortical neuronal (B50) cell line derived from an ethyl nitrosourea induced tumour in the central nervous system (CNS) of a rat was used for all cell culture experiments (Sigma Aldrich).

2.2 Methods
2.2.1 Analysis of Lupeol in Lupin Seeds
2.2.1.1 QuEChERS Extraction of Lupin Matrix from Lupin Seeds

For the determination of lupeol in lupin seeds, a QuEChERS approach was utilised to extract lupin matrix from samples of whole lupin seeds and isolated seed fractions. For the extraction of whole lupin seed, isolated seed kernels and isolated seed hull, 200.00 g of seed or seed fraction were weighed on a PL1502-S Portable Balance (Mettler Toledo, Port Melbourne, Australia) and blended for 3 minutes in a Waring Commercial Blender (Rowe Scientific, Wangara, Australia). Samples of 5.00 g of blended lupin seeds were weighed in 50 mL centrifuge tubes, to which 10 mL of water, 9 mL of acetonitrile (1% CH₃COOH volume-volume (v/v)) and 1 mL of an internal standard (ISTD) mix containing 3 deuterated polycyclic aromatic hydrocarbons (acenaphthene-d10, chrysene-d12 and phenanthrene-d10 at 1 µg.mL⁻¹ in acetonitrile) were added before manual shaking for 30 seconds. Samples were maintained on ice between all preceding extraction steps. Mixtures were homogenised for 30 seconds using an IKA T18 Basic Ultra Turrax homogeniser (Thermo Fisher Scientific Australia) before further manual shaking for 30 seconds. A single QuEChERS extraction kit as described in 2.1.1 was added to each sample, followed by manual shaking for 30 seconds. A commercial paint shaker was used to further shake samples for 1 minute. Samples were centrifuged in an Allegra X-15R Centrifuge (Beckman-Coulter Australia, Lane Cove, Australia) for 10 minutes at 5.24 x 10⁴ g at 20°C. 6.0 mL of extractant from each sample was removed and stored at -20°C for 2 hours or overnight. Samples were centrifuged for a further 20 minutes at 5.24 x 10⁴ g at -10°C. 3.0 mL of extractant was removed from each sample, to which QuEChERS clean-up kits (as described in 2.1.1) were added and manually shaken for 30 seconds. Samples were then shaken in an SO400 Paint Shaker (Fast and Fluid, Unanderra, Australia) for 1 minute before final centrifugation for 10 minutes at 5.24 x 10⁴ g at 20°C. 950 µL of supernatant matrix was removed, to which 10 µL of acetonitrile (5% v/v formic acid) was
added to acidify the matrix. 40 µL of Mirex (1 µg.mL⁻¹ in acetonitrile) was added to each extract as an ISTD for instrumental analysis. Samples were vortexed using a Vortex-Genie 2 (Scientific Industries, New York, USA) for 30 seconds before transferring 100 µL aliquots to sealed, volume-limited GC injection vials, with the remaining matrix volume stored at -4°C.

Isolated germ samples were extracted according to a modified protocol due to the limited amount of biological material available. Germ was ground to a fine powder manually using a mortar and pestle surrounded by liquid nitrogen, from which 0.500 g of germ was available. QuEChERS extraction was achieved by adding 3 mL of water and 3 mL of acetonitrile (1% CH₃COOH v/v) before manual shaking for 30 seconds. Sample was placed on ice between each step in the protocol. Germ was homogenised for 1 minute using an Omni handheld tissue homogeniser (Lomb Scientific, Taren Point, Australia) and manually shaken before the addition of a QuEChERS extraction kit as described in 2.1.1. The sample was centrifuged and frozen at the same parameters described for previous lupin samples. 1.0 mL of extract was then removed to which a clean-up kit was added as described in 2.1.1. The sample was shaken manually for 30 seconds, vortexed for 30 seconds and again shaken for 30 seconds, before centrifugation in an IEC Micromax microcentrifuge (Selby Scientific, Kewdale, Australia) at 8.12 x 10⁴ g at room temperature for 2 minutes. 475 µL of supernatant was added to 20 µL of Mirex (1 µg.mL⁻¹ in acetonitrile) with 5 µL of acetonitrile (5% v/v formic acid). Samples were analysed by vortexing the extracted matrix for 30 seconds and transferring 100 µL aliquots of matrix to sealed, volume-limited GC injection vials, with remaining matrix stored at -4°C. Peak areas determined by instrumental analysis were manually corrected for comparison to other seed fractions.

2.2.1.2 Instrumental Analysis of Lupin Matrices

Two instruments were utilised for the analysis of lupin matrices. A Bruker 450-GC coupled to a Bruker 360-MS QQQ system (Bruker Biosciences, Preston, Australia) was used for instrumental analysis of whole lupin matrices to compare QuEChERS extraction methods. Sample injection was achieved via a CP8400 autosampler equipped with a 10 µL syringe (SGE Analytical Science, Melbourne, Australia), with a cleaning mode of 5 pre-injection solvent (acetonitrile) flushes, 1
pre-injection sample flush and 5 post-injection solvent flushes. An injection volume of 1 µL of sample was used for analysis unless otherwise indicated. GC instrumentation consisted of a 1079 Programmed Temperature Vaporizer injector (Bruker Biosciences) and Siltek Split Precision inlet liner with deactivated wool (Leco). The injector temperature program consisted of an initial hold at 40°C for 1.50 minutes before increasing at 100°C.min⁻¹ to 300°C and holding for the completion of analysis. GC separation was achieved on a Restek 30 m x 0.25 mm (df = 0.25 µm) Rsi-5Sil MS capillary column with 10 m Integra-Guard column (Leco). The column oven was initially held at 40°C for 3 minutes before increasing at 30°C.min⁻¹ to 150°C and further increasing to 300°C at 11.5°C.min⁻¹, with a final hold for 10 minutes. Analyte flow through the column was constant at 1.1 mL.min⁻¹ using Ultra High Purity (UHP) helium as a carrier gas (Global Gases Australia, Balcatta, Australia). The transfer line to the MS was maintained at 250°C. MS analysis was achieved by EI at -70 eV and a source temperature of 200°C. The MS acquisition time was 31.00 minutes. In Full Scan mode, an ion detection range of m/z 45 to 600 was utilised.

A Bruker Scion-TQ GC-MS QQQ system was utilised for the comparative analysis of lupin seed fractions. Sample injection was achieved via a CP8400 autosampler equipped with a 10 µL syringe (SGE Analytical Science), with a cleaning mode of 3 pre-injection solvent (acetonitrile) flushes, 1 pre-injection sample flush and 10 post-injection solvent flushes, with an injection volume of 2 µL. GC instrumentation consisted of an 1177 Split/Splitless injector (Bruker Biosciences) and Siltek Split Precision inlet liner with deactivated wool (Leco). The injector was set to a temperature of 250°C for the duration of analysis, and was initially closed before operating at a split ratio of 100 at 1.5 minutes and 20 and 5.0 minutes. Separation was achieved on a column as described for the previous instrument. The column oven temperature program consisted of an initial hold at 80°C for 3 minutes before increasing at 30°C.min⁻¹ to 150°C and further increasing to 300°C at a at 10.0°C.min⁻¹ and holding at 10 minutes for the completion of analysis. Analyte flow through the column was constant at 1.1 mL.min⁻¹ using UHP helium as a carrier gas (Global Gases Australia). The transfer line to the MS was maintained at 270°C. MS analysis was achieved by EI at -70 eV and a source temperature of 200°C. The MS acquisition
time was 30.00 minutes. In Full Scan mode, an ion detection range of $m/z$ 50 to 600 was utilised, with standard resolution.

All chromatograms and mass spectra were inspected using MS Workstation Version 8.0 (Bruker Biosciences). Chromatographic data were investigated using Microsoft Excel for Mac 2011 version 14.3.2 (Microsoft Corporation, Santa Rosa, USA). Comparisons of lupeol extracted using different QuEChERS kits and lupeol extracted from different seed fractions were performed using a one-way analysis of variance (ANOVA) with a factor of extraction kit or seed fraction and a dependent variable of corrected lupeol peak area. Post-hoc tests for equal sample sizes were performed using a Tukey’s test for factors of extraction kit or lupin seed fraction. ANOVA and post-hoc tests were performed using SPSS version 21 for Windows (IBM, Armonk, USA).

2.2.2 Cell Culturing
2.2.2.1 Cell Culture Maintenance

B50 cells were cultured in 75 cm$^2$ filtered cell culture flasks containing DMEM supplemented with 1% (v/v) glutamine, 1% penicillin/streptomycin and 5% heat-inactivated foetal calf serum (treated at 56°C for 2 hours). Cell cultures were maintained at a constant humidified atmosphere in an Heraeus BB 15 Function Line CO$_2$ Incubator (Thermo Fisher Scientific Australia, Scoreseby, Australia) at 37°C with 5% CO$_2$. Cultures were passaged upon reaching confluence. Passaging was achieved via the removal of existing media from culture flasks followed by the addition of 2 mL of trypsin solution (0.25% trypsin in EDTA) to dislodge cells from the flask surface. Flasks were incubated for 3 minutes to ensure sufficient cell dislodgement. Trypsinisation was ceased via the addition of 8 mL of DMEM. 2 mL of cell suspension was then transferred to a new flask, to which an additional 8 mL of DMEM was added before incubation.

2.2.2.2 Cell Culture Experimentation

After passaging to generate sufficient cell suspension volume, cells were seeded onto six-well plates by adding 1 mL of cell suspension to each well, with an additional 2 mL of media. All plates were incubated for 24 hours prior to the addition of treatment or control compound
solutions to ensure confluence was reached. Compounds were diluted to required concentrations in ethanol and added to each treatment well in volumes of 30 µL. Ethanol was administered as a vehicle control in 30 µL volumes per well. Solutions were filtered through a Minisart-Plus 0.2 µm syringe filter with integral pre-filter (Supelco, Bellefonte, USA) using a 10 cc.mL⁻¹ syringe (Terumo Australia, Macquarie Park, Australia) prior to application to cell cultures. Treatment regimes were ceased by placing plates on ice and commencing cell harvesting as described in 2.2.3.1.

2.2.2.3 Cell Concentration Determination and Cell Culture Imagery

To evaluate the impact of different treatments on cell cultures, the mean number of cells present in culture was determined and compared over time, or in response to different compounds. Media was removed and cultures were incubated for 3 minutes with 500 µL of trypsin solution to dislodge cells from the well surface, followed by the addition of 2.0 mL of DMEM to cease trypsinisation. Total cell suspension was transferred to a 10 mL centrifuge tube, from which a 30 µL sample was removed and pipetted onto a Bright-Line Haemocytometer (Sigma Aldrich) for cell counting. Cells were observed through an Olympus CKX41 inverted light microscope (Olympus Australia, Mt. Waverley, Australia) and where indicated, photographed using an Olympus CKX41 microscope with Moticam 2300 camera (Micro Source Imaging, Richfield, USA) using Motic Images Plus 2.0 ML (Motic China Group, Causeway Bay, Hong Kong). Cells in the four corner squares were counted manually, with the average cell count used to calculate the number of cells present per culture well. The number of cells per well was averaged across three replicate cell culture plates to determine a mean concentration of cells per well.

The effect of treatment and control compounds on cell cultures was examined using a one-way ANOVA with a factor of time of exposure (4 or 24 hours) or compound administered (lupeol, caffeine, lupeol and caffeine or vehicle control) and a dependant variable of cells per well. Post-hoc tests for equal sample size were performed using a Tukey’s test for respective factors. ANOVA and post-hoc tests were performed using SPSS version 21 for Windows.
2.2.3 Metabolomics Analysis of Cell Cultures

Metabolomics analysis involved harvesting, extraction and derivatisation of cell and media samples, followed by instrumental analysis using GC-MS. A single cell culture plate was used to generate 1 sample of media and 1 sample of cells, with analysis performed in triplicate using three replicate plates for each treatment and control group. Analysis was conducted using 5 wells from each cell culture plate, with the remaining well used for cell imagery and/or concentration determination as described in 2.2.2.3.

2.2.3.1 Preparation of Cell Culture Samples

Cell culture plates were removed from incubation and placed on ice to commence quenching. 40 µL of media was sampled from 5 wells per plate, with samples combined and stored at -80°C. Remaining media was discarded. Cells were quenched by washing with 1 mL of PBS at 4°C followed by a second wash with 100 µL of PBS at 4°C, into which cells were manually scraped using a rubber cell scraper. Cell suspensions from 5 wells per plate were combined and stored at -80°C. Once frozen, cell suspensions and media were lyophilised overnight in a Labconco FreeZone 2.5 L Plus Cascade Benchtop Freeze Dry System (Kansas City, USA) prior to extraction.

After lyophilisation, 500 µL of a standard solution of $^{13}$C-sorbitol (2.6 µg.mL^{-1} in methanol) was added to each sample as an ISTD. Samples were resuspended by vortexing for 30 seconds. Cell samples were transferred to lysis tubes and lysed using a Precellys 24 Tissue Lyser (Sapphire Bioscience Australia, Waterloo, Australia) at $8.42 \times 10^4$ g for 2 cycles of 20 seconds. Both cell and media samples were centrifuged in an Eppendorf 5415R Centrifuge (Eppendorf South Pacific, North Ryde, Australia) at $1.32 \times 10^4$ g and 4°C for 10 minutes. 300 µL of supernatant from all samples was removed and placed under vacuum in an Eppendorf Concentrator Plus rotary vacuum concentrator for 30 minutes to visibly evaporate at least 75% of residual methanol. 300 µL of water was added to each sample before snap freezing in liquid nitrogen. Samples were lyophilised overnight prior to derivitisation.
Dervisitation was performed according to the protocol described in Gummer et al. (2012). 20 µL of methoxyamine hydrochloride solution (20 mg.mL⁻¹ in pyridine) was added to each sample, followed by agitation in an Eppendorf Thermomixer Comfort at 1200 rpm and 30°C for 90 minutes. Samples were then centrifuged at 1.32 x 10⁴ g and 4°C for 1 minute before the addition of 40 µL of MSTFA. Samples were then agitated at 300 rpm and 37°C for 30 minutes. 60 µL from each sample was then transferred to a clear injection vials for instrumental analysis, to which 5 µL of n-alkanes (6.2 µg.mL⁻¹ in hexane) was added. Samples were transferred to individual GC analysis vials containing 100 µL glass vial inserts and stored at 4°C if not analysed immediately after derivatisation.

2.2.3.2 Instrumental Analysis of Cell Culture Samples

An Agilent 6890 Series GC coupled to an Agilent 5973 Series MS (Agilent Technologies Australia, Mulgrave, Australia) with a single QMF was utilised for instrumental analysis of B50 cell and media samples. Sample injection was achieved via a an Agilent 7683 Series autosampler equipped with a 10 µL syringe (SGE Analytical Science), with a cleaning mode of 5 pre-injection solvent (methanol) flushes, 1 pre-injection sample flush and 10 post-injection solvent flushes. An injection volume of 1 µL of sample was used for analysis. GC instrumentation consisted of an Agilent Split/Splitless injector with 4.0 mm ID Split/Splitless FocusLiner (SGE Analytical Science). Separation was achieved on a 30 m x 0.25 mm (df = 0.25 µm) Agilent FactorFour VF-5ms fused silica capillary column with 10 m Integra-Guard column using UHP helium as the carrier gas. The temperature of the inlet was 230°C and the initial oven temperature was 70°C. Oven temperature was increased at 1°C.min⁻¹ for 5 minutes then increased at 5.60°C.min⁻¹ until a temperature of 330°C was reached, which was held until the completion of analysis. The transfer line to the mass spectrometer was maintained at 330°C. MS analysis was achieved in Full Scan mode with an ion detection range of 45 to 600 m/z by EI at -70 eV with a source temperature of 230°C.
2.2.3.3 Data Analysis

Data were deconvoluted and inspected using AnalyzerPro version 2.7.0 (SpectralWorks, Runcorn, UK). Individual mass spectra for chromatographic peaks were identified and compound-matched to an in-house library of metabolite standards (Murdoch University node, Metabolomics Australia). Data were exported as a matrix, inspected in Microsoft Office Excel 2003 version 11.0 (Microsoft Corporation) and manually corrected using AnalyzerPro where appropriate. Where the sample set data displayed a relative standard deviation (RSD) for the ISTD greater than 50%, uncorrected metabolite peak areas were used for analysis. Multivariate analysis (PCA) was performed with log-transformed peak areas using The Unscrambler X version 10.1 (CAMO, Oslo, Norway). PCA was conducted using a non-iterative partial least squares algorithm, cross validation, with no rotation for detection of trends between groups. Score plots were used to identify trends and loading plots to identify the metabolites influential for variation in scores plots. Metabolites with loadings value greater than 0.05 or less than -0.05 had the greatest influence on sample set variation and were utilised to compare treatment and control groups. Comparisons between groups were performed by determining mean peak areas for selected metabolites across 3 replicates and comparing means to determine if the treatment group displayed an increased (greater) or decreased (lower) peak area of each selected metabolite. Mean metabolites peak areas were determined using Microsoft Excel for Mac 2011 version 14.3.2 (Microsoft Corporation). The effect of lupeol, caffeine and lupeol with caffeine exposure was determined using independent sample T-Tests, with factors of treatment group or time of exposure and a dependant variable of metabolite peak areas of 3 replicates. T-Tests were performed using SPSS version 21 for Windows.
3. Results and Discussion
3.1 Application of the QuEChERS Approach to Lupin-Derived Lupeol

To assess the application of a QuEChERS approach to the extraction of lupeol from lupin seeds, 2 different extraction kits were utilised to extract lupin matrix from samples of whole lupin seeds. This was performed by generating 2 sample sets of lupin seeds derived from the same source, each consisting of 5 replicate 5.00 g samples of ground seeds. One sample set was extracted using a kit supplied in a 50 mL centrifuge tube, while the other was extracted using a kit supplied in a mylar pouch (described in 2.1.1). Instrumental analysis of all samples was performed in Full Scan mode using the Bruker 360 GC-MS. Total Ion Chromatograms (TICs), which illustrate the sum total of all m/z abundances detected at respective RTs with given signal intensities, were inspected for each sample. Peaks observed in TICs were identified via mass spectra comparison to the NIST (National Institute of Standards and Technology) database. Figure 3.1 illustrates a representative TIC of whole lupin seed matrix generated using a QuEChERS extraction and analysed by GC-MS.

![Figure 3.1: Representative TIC of whole lupin seed matrix analysed by GC-MS and extracted using a QuEChERS extraction kit housed in a mylar pouch. Numbered peaks correspond to compounds identified using a NIST dataset comparison of mass spectra determined at the apex of chromatographic peaks (spectra not shown). (1) acenaphthene-d10 (ISTD); (2) adipic acid; (3) capric acid; (4) phenanthrene-d10 (ISTD); (5) angustifoline; (6) \( \alpha \)-isolupanine; (7) lupanine; (8) chrysene-d12; (9) mirex (ISTD); (10) palmitoyl chloride; (11) octadecanamide; (12) octadecanedioic acid; (13) \( \gamma \)-tocopherol; (14) lupeol.](image)
As illustrated by Figure 3.1, lupeol was identified in the TIC of lupin seed samples at a RT of approximately 26.04 minutes. This was consistent across all replicates from both samples sets extracted using different QuEChERS kits.

To the knowledge of the author, only a single study has previously detected lupeol in lupin seeds (Hamama and Bhardwaj, 2004). The method utilised in Hamama and Bhardwaj (2004) consisted of a hexane/isopropanol extraction solvent (3:2, v/v) to remove a lipid fraction from ground lupin seeds, which was separated using 1-dimensional TLC with chloroform/diethyl ether (9:1 v/v) as the developing solvent. TLC produced a fraction of triterpene alcohols, which were dried and stored at -10°C before silylation and GC analysis. Compound identification was performed by chromatographic comparisons to the standard lanosterol, a structurally related triterpene. The method utilised in this work displays minor similarities to Hamama and Bhardwaj (2004), including the use of 5 g of ground lupin seeds as a starting material and analysis by GC. While the QuEChERS approach utilised in this work requires a greater number of steps in sample preparation, QuEChERS extraction is comparatively simpler, requires less solvent volume and can reduce sample loss and potential errors by coupling separation and analysis in the use of GC-MS. The method utilised in this work may therefore be more advantageous for use in the extraction of lupin-derived lupeol.

Figure 3.1 also illustrates various co-extracted compounds detected in lupin matrix. Compounds include the lupin alkaloids angustifoline, α-isolupanine and lupanine. The presence of these compounds is consistent with Greirson et al. (1991), who identified the same 3 alkaloids in L. angustifolius seed via enzyme-linked immunosorbent assay using polyclonal antialkaloid antibodies. Such lupin alkaloids have also been detected in L. angustifolius seeds by Lee et al. (2007a) and Reinhard et al. (2006) by GC-MS, in addition to the related alkaloids isoangustifoline and 13-hydroxylupanine. Reinhard et al. (2006) notably derivatised samples in a solution of bis(tri-methylsilyl)acetamide(trimethylchlorosilane/pyridine (8:1:1) prior to analysis, which improved the number of detectable alkaloids. The absence of derivatisation in this work may account for the absence of detection of additional related alkaloids, which may have been
insufficiently thermally labile for GC-MS analysis. The relatively poor signals of angustifoline and α-isolupanine as illustrated in Figure 3.1 may also reflect poor thermal stability, which could also be improved by the use of derivatisation.

Figure 3.1 also shows that γ-tocopherol was detected in lupin seed matrix with a RT of 21.67 minutes. There are multiple reports of the identification of γ-tocopherol, a vitamin E isomer with antioxidant properties, in lupin seeds using various instrumental platforms. Notable examples include work by Lampart-Szczapa et al. (2003), which utilised GC with a flame ionisation detector to identify α, δ and γ-tocopherol isomers in the seeds of L. luteus and L. albus. These identifications were consistent with the work of Martínez-Villaluenga et al. (2006), which identified the 3 tocopherol isomers in L. albus seeds via LC and Boschin and Arnoldi (2011), who detected α and γ isomers in L. angustifolius and L. albus also via LC analysis. The absence of α or δ-tocopherol in this work may be due to the clean-up protocol employed in the QuEChERS approach, which removes matrix components to ensure effective chromatographic analysis (Cunha et al., 2007).

Other components of lupin matrix exhibited by Figure 3.1 include capric acid (RT of 11.05 minutes), a 10-carbon fatty acid. Capric acid has been detected in the kernel of the legume Sphenostylis stenocarpa (African yam bean) by Adeyeye et al. (1999), in an oil fraction extracted by soxhlet extraction and analysed by GC with a flame ionisation detector. However, to the knowledge of the author, capric acid has not been previously reported in other legumes such as lupins, suggesting this may be the first identification of the compound in the Lupinus genus.

Compounds such as adipic acid (at 10.27 minutes), octadecanamide (19.65 minutes) and palmitoyl chloride (19.49 minutes) in Figure 3.1 appear to be contaminants of the matrix, as they would not normally be expected to occur in biological systems (Wishart et al., 2013). Such compounds may have been introduced during storage prior to, or during, sample preparation, or may have been incorrectly identified when spectra were compared to the NIST database.
3.2 Selection of QuEChERS Extraction Kit for Subsequent Determinations

To select a single QuEChERS extraction kit for use in subsequent work, signals of lupeol and an ISTD (mirex) in lupin seed matrix were investigated to determine which kit provided the most reproducible analysis. This comparison of kits was performed in several stages.

Firstly, 3 unique ions with different $m/z$ were selected from the mass spectra of lupeol and the ISTD (shown in Figure 3.2).

**Figure 3.2:** Representative mass spectra of (A) lupeol and (B) mirex (ISTD) generated by GC-MS analysis of whole lupin seed matrix extracted using a QuEChERS extraction kits housed in a mylar pouch. Ions tested for reproducibility in whole lupin seed samples extracted using different QuEChERS extraction kits are circled.

For lupeol, Figure 3.2 illustrates that $m/z$ 135.2, 121.2 and 95.2 were selected while $m/z$ 271.8, 236.8 and 118.9 were selected for the ISTD. For lupeol and the ISTD, the respective base peaks
of 95.2 and 271.8 were selected due to abundance in the spectra. Remaining ions were selected based on relatively high abundances in the spectra or relatively dissimilar m/z values compared to those already selected, providing a broad variety of ions for investigation.

Secondly, ions identified in Figure 3.2 were used to generate extracted ion chromatograms (EICs) of lupeol and the ISTD. An EIC differs to a TIC by displaying signal intensities of a single ion with a specific m/z, instead of all detected ions with various m/z. By utilising an ion characteristic of the compound of interest, the EIC generated is specific to the compound and provides an accurate determination of the peak area detected (and thus the amount of compound present). EICs are therefore useful in distinguishing compounds of interest from other compounds present in complex matrices, or compounds with similar RTs that exhibit ions of different m/z (Gross, 2011).

Thirdly, the generation of EICs for each ion for both compounds were used to determine peak areas of lupeol and the ISTD in all samples of lupin seed matrix. Peak areas of lupeol determined were averaged at each m/z, with the process repeated for the 3 ions of the ISTD. Results of the determination of peak areas for each compound using 3 different ions are presented in Table 3.1.

Table 3.1: Mean EIC peak area, standard deviation and relative standard deviation (RSD) (%) of lupeol and ISTD determined by GC-MS analysis of whole lupin seeds extracted with QuEChERS extraction kits.

<table>
<thead>
<tr>
<th></th>
<th>ISTD EIC Peak Area at m/z</th>
<th>Lupeol EIC Peak Area at m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>271.8</td>
<td>236.8</td>
</tr>
<tr>
<td>Mean Peak Area</td>
<td>3.66 x 10^7 ± 2.13 x 10^6</td>
<td>2.51 x 10^7 ± 1.05 x 10^6</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>5.8</td>
<td>4.2</td>
</tr>
</tbody>
</table>

As illustrated in Table 3.1, m/z 236.8 was the most reproducible ion for the ISTD, demonstrating a RSD of 4.2% (compared to 4.6% at m/z 118.9 and 5.8% at m/z 271.8). For lupeol, m/z 135.2 was the most reproducible, expressing a RSD of 18.2%. As m/z 236.8 for the ISTD and m/z 135.2 for lupeol provided the most reproducible peak areas, these target ions were utilised in further analyses to specifically identify lupeol and the ISTD respectively.
Finally, peak areas of lupeol at $m/z$ 135.2 were corrected to that of the ISTD at $m/z$ 236.8 to account for instrumental variation. Corrected lupeol EIC peak areas were then averaged across replicate samples extracted using a kit housed in a mylar pouch and compared to the mean corrected lupeol EIC peak areas of replicates extracted using the centrifuge tube kit. Figure 3.3 illustrates the comparison of mean lupeol peak areas determined using either kit.

Figure 3.3: Mean EIC peak areas with standard deviations of lupeol ($m/z$ 135.2) corrected to ISTD ($m/z$ 236.8) determined using GC-MS analysis of whole lupin seed matrix extracted using a QuEChERS extraction kit housed in a mylar pouch ($n = 6$) or 50 mL centrifuge tube ($n = 6$).

As shown in Figure 3.3, a 50 mL tube extraction kit yielded an average corrected lupeol peak area of $0.13 \pm 0.01$. Lupeol extracted using a mylar pouch extraction kit yielded a slightly greater average corrected peak area of $0.14 \pm 0.03$. The RSD of 50 mL tube kit extractions was 10.2% while in mylar pouch extractions, the RSD was greater at 23.5%. Using an independent samples T-Test, there was no statistically significant difference in the mean lupeol peak area extracted using either kit ($t(8) = 0.166, p = 0.872$). These data therefore suggested that although both kits recovered statistically similar amounts of lupeol from whole *L. angustifolius* seeds, the kit housed in a centrifuge tube provided the most reproducible analysis of lupeol. The centrifuge tube kit was therefore selected for future determinations of lupeol in *L. angustifolius* seeds and seed fractions.
3.3 Determination of Lupeol in Whole Lupin Seeds and Seed Fractions

To inform the location and relative abundance of lupeol within lupin seeds, samples of whole \textit{L. angustifolius} seeds and isolated kernel, hull and germ were extracted using the established QuEChERS approach and extraction kit housed in a 50 mL tube. Instrumental analysis of matrix was performed in Full Scan mode using the Bruker Scion-TQ GC-MS QQQ system with data analysis performed utilising EIC peak areas of lupeol at $m/z$ 135.2, corrected the ISTD at $m/z$ 236.8 to account for instrumental variation.

Figure 3.4 illustrates mean corrected peak areas of lupeol determined from lupin seed fractions, with a statistically significant difference in the amount of lupeol detected in seed fractions as determined by one-way ANOVA ($F(2,6) = 1644.468, p = <0.001$ samples).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.4.png}
\caption{Mean peak areas and standard deviations of mean correct peak area of lupeol in \textit{L. angustifolius} whole seed (n = 3), kernel (n = 3), hull (n = 3) or germ (n = 3). Matrices were extracted using a QuEChERS kit housed in a 50 mL centrifuge tube and analysed by GC-MS with peak area data generated from EICs at $m/z$ 135.2, corrected to the ISTD at $m/z$ 236.8. Statistically significant ($p = < 0.001$) differences in the mean correct peak area of lupeol relative to whole seed (*) or isolated germ (**) are identified.}
\end{figure}

A Tukey post-hoc test determined corrected lupeol peak areas were significantly greater in the isolated hull fraction (1.01 ± 0.04) compared to whole seed (0.07 ± 0.01, $p = <0.001$) and isolated germ (0.01 ± 0.01, $p = <0.001$). However, peak areas determined in whole seeds were not
significantly different to those determined in isolated germ fractions \( (p = 0.096) \). Lupeol was not
detected in any replicates of isolated lupin seed kernel. Hull samples also produced the most
reproducible analysis of all fractions with a RSD of 3.8\% compared to 15.6\% for isolated germ
samples and 19.9\% for whole seed.

Due to time constraints and confounding experimental difficulties, the development of a method
to quantitatively analyse lupeol in seed fractions could not be completed and thus, no quantitative
data is presented.

Data therefore suggested lupeol was significantly more abundant in lupin seed hulls than other
seed fractions and did not occur in seed kernels. The detection of lupeol in whole seeds could be
accounted for by the presence of lupeol in hulls and similarly, the detection of lupeol in germ
samples may be explained by the close proximity of germ tissue to the seed coat, as was
previously illustrated in Figure 1.1B. The absence of lupeol in seed kernel, however, may reflect a
potential localisation of lupeol to the seed hull, which prevents the compound from penetrating
the cotyledons.

To the knowledge of the author, this is the first report of relative abundance of lupeol within
specific fractions of lupin seeds and the first to identify a potential localisation of lupeol to the
outermost region of the seed. The abundance of lupeol in the seed hull suggests a number of
important biological considerations regarding the role of lupeol and the purpose for its relatively
high expression in seed hulls. For example, given the various pharmacological properties
exhibited by lupeol, which includes anti-bacterial, anti-viral, anti/protozoal and anti-mycotic
activities (Gallo and Sarachine, 2009), the expression of lupeol in the outermost region of the
seed may be a protective mechanism against microorganism attack. The expression of chemical
defences in the seed coat is not uncommon in plants or more specifically, legumes. Seed coats
play a fundamental role in plant reproduction by influencing seed dormancy, embryo
development and the protection of the embryo from pathogens, pests and parasites (Miao et al.,
2001). In legumes, a broad variety of compounds including alkaloids, peptides, flavonoids and
terpenes are believed to function as chemical defences and deterrents in seed coats to ensure the
viability of the internal embryo and safeguard the development of progeny (Ndakidemi and Dakora, 2003; Moïse et al., 2005). For example, Ebi et al. (2011) determined the seed coat of the legume *Detarium microcarpum* to contain multiple saponins and flavonoids and exhibit antibacterial activity against Gram-negative species including *P. aeruginosa, K. pneumonia* and *Escherichia coli* and the Gram-positive *Bacillus subtilis*. Antibacterial activity was determined by applying a 10 mg.mL\(^{-1}\) methanol extract of the seed coat to aforementioned bacterial cultures, which were inoculated with 100 µL of a 1 x 10\(^5\) cells/well broth culture to 20 mL of Mueller Hinton agar. Following 25 hours of incubation with the extracts, bacterial cultures displayed MIZs ranging from 15 to 20 mm. As previously described, lupeol extracts have also shown comparable antibacterial activities using similar experimental methods (Ahamed et al., 2007; Lutta et al., 2008; Shai et al., 2008). Lupeol may therefore function as a secondary metabolite synthesised in lupin seed hulls as a chemical defence.

### 3.4 Effect of Lupeol on B50 Cell Cultures

To examine the effects of lupeol on neuronal cell cultures, 15 replicate plates of confluent B50 cells were randomly assigned to 4 treatment groups of 3 plates. 2 groups were exposed to 10 µM lupeol, 1 of which was incubated for 4 hours and the other incubated for 24 hours. The remaining 2 groups were treated with vehicle control and also incubated for 4 or 24 hours. An additional group did not receive the vehicle control or lupeol and was utilised for determining cell concentration at the time of treatment. Following incubation, cell concentration determination and a metabolomics analysis was performed on each treatment and control group. An acute dose of 10 µM lupeol was determined to be suitable for use in this work based on various reports concluding lupeol was bioactive at this dose *in vitro* (Kim et al., 2003; Saleem et al., 2005b; Lee et al., 2007c). B50 cells utilised were selected as the cell line is established in the field of neurotoxicology and has been previously used as an effective model for investigations of neurotransmitter synthesis, neuronal cell behaviour and neurotoxicity (Bottone et al., 2008; Green, 2012; Ibegbu et al., 2012).
3.4.1 Effect of Lupeol on B50 Cell Concentration

The mean number of B50 cells present in cultures exposed to 10 µM lupeol or vehicle control for 4 or 24 hours was determined, with results presented in Figure 3.5.

![Mean Concentration of B50 Cells in Culture Over 24 Hours Following Exposure to 10 µM Lupeol](image)

Figure 3.5: Mean number of B50 rat cortical neuronal cells present per culture well after exposure to 10 µM lupeol or vehicle control for 4 or 24 hours, determined from 3 replicate cell culture plates.

Figure 3.5 illustrates that lupeol-treated and control cell cultures exhibited a decline in the average number of cells present 4 hours after treatment. 24 hours after treatment, control cells had increased in number and were greater in abundance (8.8 x 10^5 cells/well) than at the commencement of the treatment regime (7.5 x 10^5 cells/well). This would be expected to occur given the growth of cell cultures over time in the presence of adequate nutrients and growth factors supplied in DMEM. However, concentrations determined did not appear reproducible, with a standard deviation of 3.0 x 10^5 cells/well after 4 hours and 1.6 x 10^5 cells/well after 24 hours for control cultures. Lupeol-treated cultures increased in number after 24 hours but remained less abundant than at the commencement of the regime (7.2 x 10^5 cells/well after 24 hours). Despite this difference to controls, there were no statistically significant differences in the mean number of cells per well between treatments at either time interval (F(4,10) = 1.474, p = 0.281), suggesting lupeol did not affect the number of cell present in culture.
3.4.2 Effect of Lupeol on B50 Cell Culture Metabolite Profiles

Metabolomics analysis was performed on cell cultures exposed to either 10 µM lupeol or vehicle control after 4 and 24 hours to determine the effects of lupeol exposure on metabolite profiles. PCA was used to determine changes in metabolites in response to lupeol (relative to controls) at 4 and 24 hours and to compare changes in response to lupeol over time. Figures 3.6 and 3.7 illustrate PCA plots from treatment and control groups after 4 and 24 hours, derived from cells and media samples respectively.

Figure 3.6A illustrates 46% of the variation between all cell samples was described by principal component 1 (PC-1) and principal component 2 (PC-2). In Figure 3.6A, control samples are differentiated by incubation time across PC-1, illustrated by a grouping of samples incubated for 4 hours to the left of PC-1 and a group of samples incubated for 24 hours to the right. In opposition, groupings of samples exposed to lupeol for different time periods exhibit some overlapping, indicating metabolite profiles of treated cells may appear similar despite a difference in incubation time. Figure 3.6A also suggests there is poor delineation between lupeol and control samples indicated by the significant overlap of samples incubated for the same amount of time. Figure 3.6A therefore indicates that time of incubation is generally a more significant means of differentiating samples, with the majority of 4 hour incubation samples found to the right of PC-1 and all 24 hours incubation samples found to the left.

Figure 3.7A illustrates that PC-1 and 2 explain 58% of the variance between all media samples, a value greater than that explained by PCs for cell samples (46% in Figure 3.6A). Figure 3.7 shows that media samples of control cell cultures were not completely separated by either PC while media samples from lupeol-treated cell cultures were clearly differentiated by PC-1. This is in opposition to the overlap of lupeol-treated samples observed in Figure 3.6A. Figure 3.7A also illustrates overlap between groupings of lupeol-treated media samples incubated for 24 hours, with all control samples, suggesting these samples display similar metabolite profiles. In opposition, the grouping of lupeol treated media samples incubated for 4 hours does not overlap with any group, suggesting these samples are less similar to all other media samples.
Figure 3.6: PCA plots of cell samples of B50 rat cortical neuronal cell cultures exposed to 10 µM lupeol for 4 (n = 3) or 24 hours (n = 3), or vehicle control for 4 (n = 3) or 24 hours (n = 3) and analysed by GC-MS. (A) Scores plot of samples situated according to relative intersample similarity with identified groupings of replicates. (B) Loadings plot of metabolites identified within the sample set positioned according to relative influence on total intersample variation.
Figure 3.7: PCA plots of media samples of B50 rat cortical neuronal cell cultures exposed to 10 µM lupeol for 4 (n = 3) or 24 hours (n = 3), or vehicle control for 4 (n = 3) or 24 hours (n = 3) and analysed by GC-MS. (A) Scores plot of samples situated according to relative intersample similarity with identified groupings of replicates. (B) Loadings plot of metabolites identified within the sample set positioned according to relative influence on total intersample variation.
Further, Figure 3.7 again indicates that time of incubation is a more significant factor in differentiating samples, rather than whether or not samples were administered lupeol, with the majority of 4 hour incubation samples found to the left of PC-1 and all 24 hours incubation samples appearing to the right, while lupeol samples appear evenly distributed across both PCs.

Figures 3.6 and 3.7 therefore indicate lupeol exerted minimal biochemical impact on the metabolite profiles of B50 cells over 4 and 24 hours. In contrast, media samples exposed to lupeol initially exhibited metabolic differences following lupeol exposure. However, these differences were normalised over time, as extracellular samples became more similar to controls after 24 hours. Figures 3.6 and 3.7 may therefore indicate the compound is transported into the cell and rapidly metabolised, is retained by the cell but displays minimal biological effect or degrades in the media between 4 and 24 hours. These results therefore suggest the metabolic consequences of lupeol are minimal and/or only observable for a short timeframe.

**3.4.2.1 Effect of Lupeol on B50 Cell Culture Metabolite Profiles after 4 Hours**

PCA was used to examine the impact of lupeol on B50 cell culture metabolite profiles after 4 hours, relative to cell cultures incubated for the same time with a vehicle control. Scores plots of lupeol-treated and control cell and media samples are presented in Figure 3.8.

Figure 3.8 illustrates 65% of the variation between cell samples incubated for 4 hours was described by PC-1 and 2. This is less than the 82% variance described by PCs modelling the media samples incubated for the same time period. Figure 3.8A indicates the metabolite profiles of cell samples are relatively unreproducible within treatment and control groups. There is also a notable absence of complete separation by either PC, with samples from the control and treatment groups dispersed broadly across the plot. In contrast, Figure 3.8B illustrates media samples from both groups were clearly differentiated by PC-2. Lupeol-treated media samples also appeared reproducible, as suggested by a tight clustering of samples while control media samples were again broadly distributed across the plot (Figure 3.8B). Figure 3.8 therefore indicates the administration of lupeol resulted in greater dissimilarities in metabolite profiles of media samples,
than cell samples and effected minimal change to the intracellular metabolite profiles of cells relative to controls.

**Figure 3.8:** PCA plots of B50 rat cortical neuronal cell cultures exposed to 10 μM lupeol (n = 3) or vehicle control (n = 3) for 4 hours and analysed by GC-MS. (A) Scores plot of cell samples situated according to relative intersample similarity with identified groupings of replicates. (B) Scores plot of media samples situated according to relative intersample similarity with identified groupings of replicates.
From Figure 3.8, metabolites exerting the greatest influence on variation between samples analysed after 4 hours were selected for further investigation. The peak areas of selected metabolites were averaged across 3 replicates in each treatment group and compared directly to determine the relative impact of lupeol (Table 3.2).

Table 3.2: Differences in metabolites determined by PCA as most influential on the variance of samples from B50 rat cortical neuronal cell cultures exposed to 10 µM lupeol (n = 3) relative to vehicle control samples (n = 3) for 4 hours. Metabolites are presented by sample type and metabolite class, where “↑” indicates greater metabolite levels observed in lupeol-treated samples relative to controls and “↓” indicates lower metabolite levels in lupeol-treated samples relative to controls.

<table>
<thead>
<tr>
<th>Intracellular Samples</th>
<th>Extracellular Samples</th>
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</thead>
<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td><strong>Amino Acids</strong></td>
</tr>
<tr>
<td>Proline</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td></td>
</tr>
<tr>
<td><strong>Carbohydrates</strong></td>
<td><strong>Carbohydrates</strong></td>
</tr>
<tr>
<td>Galactose</td>
<td>Galactose</td>
</tr>
<tr>
<td>Unidentified Carbohydrate</td>
<td>Gluconate</td>
</tr>
<tr>
<td>Mannose</td>
<td>Lactate</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>2 Unidentified Carbohydrates</td>
</tr>
<tr>
<td>Lactose</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
</tr>
<tr>
<td>Unidentified Carbohydrate</td>
<td></td>
</tr>
<tr>
<td><strong>Fatty Acids</strong></td>
<td><strong>Fatty Acids</strong></td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>Oleic Acid</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>GABA</td>
<td></td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td></td>
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<tr>
<td>Urea</td>
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Table 3.2 illustrates metabolites of varying classes affected by lupeol administration, where none of the reported differences in response to lupeol were statistically significant. For example, α-amino acids generally decreased in treated cells, as observed by reductions in arginine, cysteine and tyrosine. In extracellular samples, only tyrosine was found to influence sample set variation and appeared to increase in abundance. This may suggest increased tyrosine exportation from cells into the media, or increased utilisation of intracellular amino acids for biochemical processes. For example, tyrosine may be converted to compounds such as the neurotransmitter...
dopamine, via tyrosine hydroxylase. Dopamine may also be further converted to norepinephrine and epinephrine, all of which are important in both central and peripheral nervous system signal transduction (Daubner et al., 2011). As the cell line utilised was derived from a rat CNS, lupeol may be modulating neurotransmitter metabolism and therefore affecting intracellular tyrosine utilisation.

Cysteine may be readily oxidised to a reduced form of glutathione (GSH), a compound with numerous metabolic functions that was reported to induce neuronal differentiation in rat bone marrow cells (Stipanuk, 2004; Sagara and Makino, 2008). Given that cultures utilised in this work expressed cells with either a rounded or elongated morphology, the latter form displaying process-like extensions, neuronal cell differentiation may be a metabolic fate of cysteine which lupeol is up-regulating.

GSH is also involved in antioxidant activity, xenobiotic metabolism and the maintenance of thiol redox state in the CNS (Aoyama et al., 2008). Lupeol may therefore be modulating the intracellular use of GSH for these metabolic processes instead, or in addition to, cellular differentiation. This would be consistent with several reports investigating the mechanism of antioxidant activity of lupeol, which demonstrates a modulation of GSH levels and other enzymatic and non-enzymatic antioxidants to perform bioprotective functions against oxidative stress (Saleem et al., 2001; Preetha et al., 2006; Gupta et al., 2012; Kumari and Kakkar, 2012). For example, Saleem et al. (2001) determined topical application of lupeol to murine skin (0.75 to 1.5 mg per animal) 1 hour prior to exposure to 20 mg benzoyl peroxide, a tumour promoter inducing oxidative stress, prevented the induced inhibition of GSH dose-dependently. Lupeol may therefore modulate GSH synthesis in treated-cells, resulting in GSH utilisation and observed declines in cysteine.

In contrast to the trend of amino acid decline, proline was more abundant in cell samples (Table 3.2). Increased intracellular proline may be the result of reduced metabolism or stimulation of a proline transport system. Several proline transport systems are known to exist in rat neuronal tissues, including a high affinity Na⁺ and Cl⁻-dependant plasma membrane proline transporter.
(PROT) specific to proline (Valez-Faircloth et al., 1995) and systems capable of transporting multiple amino acids with proline including cysteine, alanine, serine and glycine, which may be stimulated by intracellular Ca$^{2+}$ and Ca$^{2+}$-mobilising agents (Zafra et al., 1994). As lupeol has been previously shown to interact with intracellular signalling pathways and secondary messengers including the phosphatidylinositol 3-kinase/Akt, mitogen activated protein kinase and nuclear factor-κB pathways as an anti-cancer agent in human pancreatic adenocarcinoma cells (Saleem et al., 2004), lupeol may interact with signaling pathways utilising Ca$^{2+}$ mobilisation, resulting in proline influx. Alternatively, lupeol may play a more direct role in modulating proline uptake via the high affinity PROT.

A number of modifications were also observed in carbohydrate levels in response to lupeol exposure after 4 hours (Table 3.2). Changes were variable across the metabolite class, with galactose and mannose increasing, while dulcitol, sucrose and lactose decreased intracellularly. Extracellular samples exhibited a general decrease in carbohydrate levels with declines in the amounts of galactose, gluconate and lactate. The trend of extracellular carbohydrate decrease may indicate an influx of carbohydrates from the media into the cell. This is particularly evidenced by the decrease of galactose in media samples with an associated increase in cell samples (Table 3.2). Galactose can eventually be converted to glucose-6-phosphate, which can then be utilised in the production of cellular adenosine triphosphate (ATP) via glycolysis and subsequently, the citric acid cycle. Similarly, mannose is converted to fructose-6-phosphate, another intermediate of glycolysis (Garrett and Grisham, 2013). Neuronal cells are also known to produce lactate when activated and may potentially utilise lactate as an energy source (Dienel and Hertz, 2001). These data therefore indicate the impact of lupeol on carbohydrate metabolism and more specifically, the glycolytic pathway, may extend to a modulation of utilising multiple energy sources.

Intracellular γ-Aminobutyric acid (GABA), an inhibitory neurotransmitter of the mammalian CNS, was also comparatively more abundant in response to lupeol treatment (Table 3.2). Similarly to changes observed in tyrosine, lupeol may modulate the synthesis of neurotransmitters leading to an intracellular increase in GABA production.
Table 3.2 also presents a decrease in urea in lupeol-treated cell samples. The change in urea may indicate potential interactions with enzymes or intermediates involved in the urea cycle, a biochemical pathway utilised for the removal of nitrogen from the cell achieved by several reactions occurring within the mitochondrion and cytosol. Changes in the urea cycle are reinforced by the observed decrease in arginine, which in the cytosol, is converted to urea enzymatically via arginase (Campbell and Farrell, 2012). Lupeol has been previously shown to moderate levels of urea in a report by Sudhahar et al. (2008), investigating the activity of lupeol as a cardio- and nephroprotective agent against hypercholesterolaemia, which may lead to atherosclerosis and renal dysfunction (Balarini et al., 2011). Using a rat model for induced hypercholesterolaemia, Sudhahar et al. (2008) demonstrated animals fed a high cholesterol diet for 30 days showed significantly increased urea in serum (36.45 mg.dl⁻¹) relative to animals receiving a normal diet (26.25 mg.dl⁻¹). However, rats receiving 50 mg lupeol per kg body weight per day for 15 days, whilst receiving a high cholesterol diet, displayed urea levels not significantly different to rats receiving the normal diet (30.79 mg.dl⁻¹) but significantly different to those receiving the high cholesterol diet without lupeol. Lupeol may therefore modulate intracellular levels of urea or affect rates of urea production, however a mechanism for this activity has not been elucidated.

Changes in fatty acids in lupeol-treated cells incubated for 4 hours were complimentary between media and cell samples, with an observed decrease in intracellular fatty acids (exemplified by the 18-carbon essential polyunsaturated ω-6 fatty acid linoleic acid) and an increase in fatty acids in the media (exemplified by the 18-carbon monounsaturated Δ⁹ fatty acid oleic acid) (Nelson and Cox, 2008) (Table 3.2). The differences in fatty acids may indicate an active efflux from cells into the media due to a lupeol-induced modulation of cellular activities. For example, Sudhahar et al. (2008) also reported significantly less free fatty acids (2.35 mg.g⁻¹ tissue) and triglycerides (6.17 mg.g⁻¹ tissue) in serum of rats receiving lupeol treatment with a high cholesterol diet relative to those not receiving lupeol (3.11 mg.g⁻¹ tissue and 7.07 mg.g⁻¹ tissue respectively). Further, free fatty acid and triglyceride levels in serum were not significantly different to animals not receiving lupeol or the high cholesterol diet, suggesting the addition of lupeol with the diet prevented the
increase in fatty acid levels resulting from increased cholesterol. Lupeol may therefore modify levels of triglycerides and fatty acids intracellularly, however a mechanism for this activity has not been elucidated.

Table 3.2 also illustrates a decline in pantothenic acid (vitamin B₅) in cell samples exposed to lupeol for 4 hours. Pantothenic acid is a component of coenzyme A (CoA), notable for its role in the citric acid cycle in the oxidation of pyruvate to acetyl-CoA and α-ketoglutarate to succinate. CoA has several biochemical functions and is concerned with the synthesis of essential fatty acids, cholesterol and the neurotransmitter acetylcholine (Szutowicz et al., 2000; Jaroenporn et al., 2008). A decrease in pantothenic acid could therefore represent an increase in CoA synthesis and consequently an increase in the utilisation of the coenzyme for the aforementioned biochemical processes. This appears to support the finding that lupeol may up-regulate neurotransmitter biosynthesis or modulate the synthesis or utilisation of fatty acids and carbohydrates.

### 3.4.2.2 Effect of Lupeol on B50 Cell Culture Metabolite Profiles after 24 Hours

PCA was used to examine the metabolic effect of lupeol on B50 cell culture metabolite profiles after 24 hours, relative to cell cultures incubated for the same time with a vehicle control. Scores plots of lupeol-treated and control cell and media samples are presented in Figure 3.9.

Figure 3.9 illustrates 74% of the variation between cell samples incubated for 24 hours can described by PC 1 and 2. This is less than the 79% variance described by PCs modelling the media samples incubated for sample time period. Figure 3.9A indicates there is an absence of complete separation by either PC, with samples from the control and treatment groups dispersed broadly across the plot, consistent with the scores plot of cell samples incubated for 4 hours (Figure 3.8A). Similarly, the scores plot of media samples (Figure 3.9B) illustrates replicates were relatively unreproducible and not clearly separated by either PC. Overlap of control and treatment samples is also evident, suggesting metabolite profiles of controls were similar to the profiles of some lupeol-treated samples. This may suggest changes in response to lupeol were
primarily evident within the cell after 24 hours of treatment, whilst the media was more consistent between control and treatment groups.

**Figure 3.9:** PCA plots of B50 rat cortical neuronal cell cultures exposed to 10 μM lupeol (n = 3) or vehicle control (n = 3) for 24 hours and analysed by GC-MS. (A) Scores plot of cell samples situated according to relative intersample similarity with identified groupings of replicates. (B) Scores plot of media samples situated according to relative intersample similarity with identified groupings of replicates.
Metabolites most influencing the intersample variation of the 24-hour incubation sample set were further examined and compared to determine those increasing or decreasing in lupeol-treated samples relative to controls (Table 3.3).

**Table 3.3**: Differences in metabolites determined by PCA as most influential on the variance of samples from B50 rat cortical neuronal cell cultures exposed to 10 μM lupeol (n = 3), relative to vehicle control samples (n = 3) for 24 hours. Metabolites are presented by sample type and metabolite class, where “↑” indicates greater metabolite levels observed in lupeol-treated samples relative to controls and “↓” indicates lower metabolite levels in lupeol-treated samples relative to controls.

<table>
<thead>
<tr>
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<th>Extracellular Metabolites</th>
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<td><strong>Amino Acids</strong></td>
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<td></td>
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<tr>
<td>Urea</td>
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</table>

Table 3.3 illustrates a number of alterations to metabolites in lupeol-treated cells and media samples. Similarly to Table 3.2, which investigated changes in metabolites after 4 hours, the reported changes observed after 24 hours were not statistically significant. Amino acids were generally less abundant in cells relative to controls (Table 3.3), a result consistent with cell samples incubated with lupeol for 4 hours (Table 3.2). While the change in tyrosine could again be a result of utilisation in neurotransmitter synthesis (Daubner et al., 2011), the decline in arginine may not be a result of urea cycle inhibition as previously suggested, due to the observation that urea was more abundant in treated cells relative to controls. The absence of a statistically significant difference, however, complicates the ability to exclude an inhibition or modulation of the urea cycle as resulting from lupeol application.

Table 3.3 also illustrates extracellular proline was more abundant in lupeol-treated cultures than controls after 24 hours. This finding is also in opposition to the analysis of cells after 4 hours of...
treatment (Table 3.2), where intracellular proline was more abundant in treated cells relative to control cells. This difference in proline abundance indicates a dynamic impact of lupeol on proline transport. However, the previously described PROT may also operate bidirectionally (Valez-Faircloth et al., 1995). The effects of lupeol on PROT may therefore differ over time.

Carbohydrate levels were again mixed in response to lupeol after 24 hours (Table 3.3), with cell samples exhibiting increased glutarate and lactose, while citrate decreased relative to controls. In media samples, galactose increased while glutarate and lactate decreased in response to lupeol. The observation of lactate is consistent with the findings of media samples treated for 4 hours (Table 3.2), suggesting lupeol-induced modulations of lactate uptake for carbohydrate metabolism. Notable differences in carbohydrates between Tables 3.2 and 3.3 include intracellular lactose, which was less abundant in cells relative to controls after 4 hours but more abundant in cells relative to controls after 24 hours. This may suggest an intracellular accumulation of lactose over time in response to lupeol. Similarly, galactose was more abundant than controls in the media of samples incubated for 24 hours, despite being less abundant in the media of samples incubated for 4 hours than controls. This may suggest galactose metabolism was up-regulated over time in control cells, increasing galactose uptake into the cell, whilst lupeol-treated cells continued to utilise other energy sources. The decreased abundance of citrate in lupeol-treated indicated the citric acid cycle was actively utilising carbohydrates for ATP production. However, α-ketoglutarate, the product of citrate metabolism in the citric acid cycle, is also a precursor to the synthesis of the neurotransmitter glutamate. Since glutamate synthesis has been shown to be up-regulated in rat neuronal cell cultures (Hertz and Zielke, 2004), lupeol may dynamically impact the citric acid cycle for both energy production and glutamate synthesis.

Additional changes in response to lupeol exposure for 24 hours (Table 3.3) consisted of elevated intracellular GABA, consistent with observations in cells treated with lupeol for 4 hours (Table 3.2). Lupeol may therefore elicit a sustained increase in GABA synthesis over time. In contrast, fatty acid changes were not consistent with the previous analysis of the impact of lupeol after 4 hours (Table 3.2), demonstrating both an intracellular and extracellular increase in fatty acids.
after 24 hours of exposure. It was previously proposed lupeol modulated fatty acid metabolism, evidenced by an increase in fatty acid efflux from cells (Table 3.2). The intracellular increase in fatty acids after 24 hours (Table 3.3) may therefore indicate a more dynamic modulation of fatty acid metabolism, impacted upon variably over time.

3.4.2.3 Effect of Lupeol on B50 Cell Culture Metabolite Profiles Over Time

PCA was used to examine the metabolic effect of lupeol on B50 cell culture metabolite profiles after 24 hours of incubation, relative to cell cultures incubated for 4 hours. Scores plots of lupeol-treated and control cell and media samples are presented in Figure 3.10.

Figure 3.10A illustrates treatment groups were not clearly differentiated, indicating the metabolite profiles of lupeol-treated samples were similar between 4 and 24 hours. In contrast, Figure 3.10B shows clear separation between samples incubated for different time period. Figure 3.10 therefore indicates metabolite profiles of lupeol-treated media changed over time, while metabolite profiles of cell samples did not change over time to the same extent.

Metabolites most influencing the intersample variation of lupeol-treated samples were further examined and compared to determine those increasing or decreasing over time, as illustrated in Table 3.4. Table 3.4 illustrates a number of alterations to metabolites in lupeol-treated cells and media samples over time. Cultures incubated with lupeol displayed increased intracellular and decreased extracellular proline over time, indicating a net influx of proline. An increase in intracellular proline is consistent with observations of the metabolite increasing in intracellular abundance after 4 hours of exposure (Table 3.2) but in contrast to the increased extracellular proline observed after 24 hours (Table 3.3). Data may therefore indicate that while proline influx generally increased over time, control cells may have increased influx to a greater extent, thereby accounting for a comparatively greater abundance of extracellular proline in lupeol-treated cultures at 24 hours. Modifications in proline abundance may reflect dynamic modulations of transport mechanisms such as the PROT, or alterations to intracellular Ca$^{2+}$ consequentially impacting other proline transporters (Zafra et al., 1994; Valez-Faircloth et al., 1995).
Figure 3.10: PCA plots of B50 rat cortical neuronal cell cultures exposed to 10 µM lupeol for 4 (n = 3) or 24 hours (n = 3) and analysed by GC-MS. (A) Scores plot of cell samples situated according to relative intersample similarity with identified groupings of replicates. (B) Scores plot of media samples situated according to relative intersample similarity with identified groupings of replicates.
Tyrosine levels were also altered over time in response to lupeol, observed in lower abundance in treated cells than controls after 4 and 24 hours (Tables 3.2 and 3.3 respectively). Over time, tyrosine decreased in lupeol-treated cells (Table 3.4), potentially suggesting utilisation increased over time and that utilisation was greater in treated cells than controls. Again, the most significant biochemical pathway for tyrosine utilisation in the cell line is neurotransmitter synthesis (Daubner et al., 2011). Further analysis of the data illustrated tyrosine accumulated over time in control cells suggesting lupeol may therefore modulate neurotransmitter synthesis by increasing tyrosine utilisation.

Intracellular carbohydrates generally decreased over time (Table 3.4), where citrate was significantly less abundant after 24 hours relative to abundances at 4 hours ($t(4) = -6.47$, $p = 0.003$). A trend of carbohydrate utilisation would normally be expected to occur in an active cell culture due to requirements of ATP production for growth, development and cellular differentiation (Bottone et al., 2008). Further analysis of the data indicated lactose decreased over time in control cells, while galactose also decreased over time in control media samples,
suggesting such carbohydrates were transported into the cell and subsequently metabolised to generate ATP. While utilisation of lactose over time was also evident in lupeol-treated cells (Table 3.4), signifying this was a normal behaviour of the cell line, galactose increased in the media of lupeol-treated samples (Table 3.4). Lupeol may therefore modulate the efflux and/or utilisation of substrates for energy production. In contrast, intracellular arabitol and an unidentified carbohydrate increased in abundance over time in response to lupeol (Table 3.4). The intracellular increases in carbohydrates may therefore further suggest a modulation of carbohydrate influx or utilisation over time in response to lupeol.

Table 3.4 also illustrates a decrease in the abundance of urea over time. However, inspection of the data revealed the same trend to occur in control cell samples over time, suggesting this outcome may have occurred independently of the application of lupeol.

Extracellular fatty acids decreased in abundance in treated cultures over time, exemplified by oleic acid in Table 3.4, whilst increasing in intracellular abundance over time, as exemplified by linoleic acid. Differences in fatty acid levels therefore suggested an influx of fatty acids over time, potentially for use as an alternate energy source via β-oxidation in the mitochondrion, resulting in the production of acetyl-CoA moieties that enter the citric acid cycle for ATP production (Nelson and Cox, 2008). The influx and subsequent catabolism of fatty acids has been previously reported in cultured mouse superior cervical ganglion neurons (Tanenaka et al., 2003), suggesting this process would normally occur in rat neuronal cell cultures. Inspection of the data confirmed linoleic acid increased over time in control cell samples, suggesting the increase in Table 3.4 of intracellular linoleic acid occurred independently of lupeol. Lupeol may therefore increase fatty acid uptake, as a decline in extracellular oleic acid was not observed over time in control cells.

3.5 Lupeol as a Potential Neuroprotectant

Lupeol was investigated for potential neuroprotective properties against insult by the neurotoxic alkaloid caffeine. A dose of 1 mM caffeine was utilised following Green (2012), determining the
dose to visibly induce cellular toxicity in vitro in the same cell line. To examine the ability of lupeol to act as a neuroprotectant against caffeine, 12 replicate cell culture plates of confluent B50 cells were randomly assigned to 4 treatment groups of 3 plates. Treatment groups consisted of exposure to 10 µM lupeol, 1 mM caffeine, 10 µM lupeol and 1 mM caffeine or vehicle control, for 24 hours. Representative images were taken of cultures from each group at the conclusion of incubation, followed by cell concentration determination and metabolomics analysis.

### 3.5.1 Cell Imagery of Lupeol as a Potential Neuroprotectant

To observe differences in cell culture appearance between treatment and control groups, representative photographs of each group were captured at the conclusion of incubation and are presented in Figure 3.11.

**Figure 3.11:** Photographs of B50 rat cortical neuronal cell cultures taken at 10x magnification after incubation for 24 hours with (A) vehicle control, (B) 10 µM lupeol, (C) 1 mM caffeine or (D) 10 µM lupeol and 1 mM caffeine.
Control cell cultures appeared confluent with mixed cell morphologies, consisting of rounded cells and elongated cells with process-like extensions (Figure 3.11A). Control cultures also exhibited some aggregations of rounded cells (white arrowhead in Figure 3.11A). Cultures exposed to lupeol were similar in appearance to control cell cultures, also expressing confluent mixed cell morphologies with a similar number of rounded cell aggregations. Cultures exposed to caffeine did not appear confluent (Figure 3.11C) and contained fewer cells relative to both the control (Figure 3.11A) and lupeol-treated cultures (Figure 3.11B). Caffeine-treated cultures also exhibited a greater number of cellular aggregations and comparatively fewer elongated cells than the control and lupeol-treated cultures. Cultures co-administered caffeine and lupeol (Figure 3.11D) appeared more similar to caffeine-treated than the control or lupeol-treated cultures, evidenced by a lack of confluency and a reduced number of cells. Cultures receiving lupeol and caffeine also displayed comparatively fewer elongated cells and a greater number of aggregations of rounded cells than other treatment groups (white arrowheads in Figure 3.11D).

Figure 3.11 indicates the application of 10 μM lupeol alone did not result in visible cell damage or reduce cell concentration, indicating the compound was non-toxic at the dose applied. However, the application of 1 mM caffeine reduced the number of visible cells and resulted in cellular aggregation, suggesting caffeine was toxic to cell cultures and resulted in cellular necrosis or apoptosis. Similar findings have been described by Kang et al. (2002), reporting caffeine at doses greater than 300 μM to cultured confluent murine cortical neurons for 24 hours induced visible cell shrinkage and neuronal death. Kang et al. (2002) also reported the anti-apoptotic drug cycloheximide attenuated caffeine toxicity, signifying apoptotic pathways mediated caffeine toxicity. Caffeine-induced apoptosis may therefore account for the observed decline in cell concentration in Figures 3.11C and 3.11D. Green (2012) also determined 1 mM caffeine to visibly reduce the number of cells present using confluent B50 cells, further suggesting caffeine induced neuronal cell death.
The appearances of cultures receiving both lupeol and caffeine suggested lupeol did not afford cellular neuroprotection against caffeine toxicity. The administration of both compounds may have also increased cellular toxicity, evidenced by increased cellular aggregation (Figure 3.11D). The absence of published works exploring similar activities of lupeol prevents the comparison of this result to existing data. This report may therefore be the first of lupeol failing to protect a neuronal cell culture from toxic insult. However, previous explorations of the anti-cancer activity of lupeol in vitro using prostate or skin cancer cell lines reported lupeol demonstrated inhibition of apoptotic pathways by restricting the activity or expression of pro-apoptotic intracellular mediators including caspase-3, 6, 8 and 9, Bax and apoptotic protease activating factor-1 (Saleem et al., 2005b; Prasad et al., 2008b; Saleem et al., 2008; Nigam et al., 2009). Although the apoptotic mediators of caffeine toxicity are not currently known, lupeol may be expected to demonstrate neuroprotection by inhibition of caffeine-induced apoptosis. The lack of neuroprotection afforded may therefore not be due an inability of lupeol to provide protection against caffeine. Rather, the absence of protection may be due to an overwhelmingly acute dose of caffeine utilised, or conversely, that the dose of lupeol administered was too low.
3.5.2 Effect of Lupeol as a Potential Neuroprotectant on B50 Cell Concentration

The mean number of B50 cells present in cultures exposed to lupeol, caffeine, lupeol and caffeine or vehicle control for 24 hours was determined, with results presented in Figure 3.12.

![Mean Concentration of B50 Cells in Cultures Exposed to Lupeol and/or Caffeine for 24 Hours](image)

**Figure 3.12:** Mean number of B50 rat cortical neuronal cells present per culture well after exposure to 10 µM lupeol (n = 3), 1 mM caffeine (n = 3), 10 µM lupeol and 1 mM caffeine (n = 3) or vehicle control (n = 3) for 24 hours.

Cultures treated with only lupeol displayed the greatest number of cells (1.0 x 10⁶ ± 1.9 x 10⁵ cells/well) while cultures treated with both lupeol and caffeine displayed the lowest cell concentration (6.6 x 10⁵ ± 3.3 x 10⁵ cells/well). Intermediate cell concentrations were observed in cultures treated with vehicle control (7.4 x 10⁵ ± 6.7 x 10⁴ cells/well) or caffeine only (8.0 x 10⁵ ± 1.8 x 10⁵ cells/well). Using a one-way ANOVA, it was determined there were no statistically significant differences in the number of cells per well between any treatment group (F(3,8) = 1.755, p = 0.233).

Figure 3.12 indicates lupeol increased the number of cells present in culture however the increase was not statistically significant. The analysis of cells treated with only lupeol in Figure 3.12 is therefore consistent with the previous cell determinations of cultures treated with the same dose.
of lupeol (Figure 3.5), where lupeol administration did not result in a significant change in the number of cells present.

The application of caffeine reduced the number of cells present but again the difference was not significant. This appears inconsistent with observations of Figure 3.11, where caffeine visibly reduced the number of cells in culture. However, this disagreement could be the result of inconsistencies in the dispersal of visible cells across individual cell culture wells or between replicate plates, which were compensated for in cell concentration determination.

Co-administration of lupeol and caffeine further reduced the number of cells present relative to controls however the reduction was also not significant. This is also inconsistent with observations from Figure 3.11, where cultures were visibly sparser than controls and lupeol-treated cultures. However, given the appearance of cultures treated with both compounds was similar to those receiving only caffeine, the lack of a statistically significant difference between the two treatment groups exposed to caffeine is expected. It is therefore difficult to state whether the co-administration of lupeol with caffeine resulted in a potentiation of the toxicity of caffeine by reducing the number of cells present, or whether lupeol simply failed to protect cell cultures against caffeine toxicity.

3.5.3 Metabolomics Analysis of Lupeol as a Potential Neuroprotectant

Metabolomics analysis was performed on cell cultures exposed to 10 µM lupeol, 1 mM caffeine, 10 µM lupeol with 1 mM caffeine or vehicle control for 24 hours, to determine the effects of exposure on metabolite profiles. Using PCA, comparisons between treatment groups and controls were made to determine if compounds resulted in differences in metabolite profiles. Figure 3.13 illustrates scores and loadings plots of cells treated with lupeol, caffeine, lupeol and caffeine or vehicle control. Figure 3.14 illustrates scores and loadings plots of media treated with lupeol, caffeine, lupeol and caffeine or vehicle control, generated via PCA analysis.
Figure 3.13: PCA plots of cell samples of B50 rat cortical neuronal cell cultures exposed to 10 µM lupeol (n = 3), 1 mM caffeine (n = 3), 10 µM lupeol and 1 mM caffeine (n = 3) or vehicle control (n = 3) for 24 hours and analysed by GC-MS. (A) Scores plot of samples situated according to relative intersample similarity with identified groupings of replicates. (B) Loadings plot of metabolites identified within the sample set positioned according to relative influence on total intersample variation.
Figure 3.14: PCA plots of media samples of B50 rat cortical neuronal cell cultures exposed to 10 µM lupeol (n = 3), 1 mM caffeine (n = 3), 10 µM lupeol and 1 mM caffeine (n = 3) or vehicle control (n = 3) for 24 hours and analysed by GC-MS. (A) Scores plot of samples situated according to relative intersample similarity with identified groupings of replicates. (B) Loadings plot of metabolites identified within the sample set positioned according to relative influence on total intersample variation.
Figure 3.13A illustrates 58% of the variation between cell samples was described by 2 PCs. Figure 3.13A also illustrates lupeol-treated cells are the most reproducible of all groups, as represented by the closest aggregation of samples. Lupeol-treated and vehicle control cells are also relatively similar in metabolite profiles. This is consistent with Figures 3.8 and 3.9, investigating the impact of the same concentration of lupeol on B50 cells over 4 and 24 hours respectively. Caffeine-treated cells also demonstrated similarity in metabolite profiles to both controls and lupeol-treated cells (Figure 3.13A). The similarities in profiles may be considered in opposition to Figure 11, which displayed visible differences in cell culture appearance in response to caffeine relative to controls and lupeol-treated cultures. However, similarities in profiles of caffeine, controls and lupeol-treated cells may be due to the presence of samples treated with both lupeol and caffeine being included in the analysis, as these replicates were noticeably dissimilar to all other groups (Figure 3.13A).

PCs describing the media samples (Figure 3.14) explain less variance (56%) compared to cell samples (58%) (Figure 3.13). Figure 3.14 again indicates lupeol-treated samples are the most reproducible of all groups. In opposition to Figure 3.13, lupeol-treated media samples display less similar metabolite profiles to controls and caffeine-treated cells. Media samples treated with caffeine, lupeol and caffeine or vehicle control therefore display some similarities in metabolite profiles as identified by the overlap of samples from these groups (Figure 3.14A).

Figures 3.13 and 3.14 suggest the neurotoxic agent caffeine impacts cell and media metabolite profiles. However, treatment with both lupeol and caffeine resulted in the greatest change in metabolite profiles in cell samples. In media, samples treated with both lupeol and caffeine appeared relatively similar to the media of control samples, suggesting the impact of administering both agents primarily resulted in intracellular changes. However, lupeol only treatment resulted in the greatest change in media profiles but had similar impacts on cells to those treated with vehicle control.
3.5.3.1 Effect of Lupeol on Metabolite Profiles of B50 Cell Cultures

As the impact of acute lupeol administration at 10 µM for 24 hours has been investigated and discussed, results of a comparison of lupeol-treated and control cell cultures in this experiment are not presented. PCA and a comparison of metabolites in lupeol-treated intracellular and extracellular samples relative to controls largely reflected results previously described, with no clear trends in amino acids, carbohydrates and fatty acids. It should be noted that the only significant difference of lupeol-treated samples relative to controls in this experiment was a reduced abundance of intracellular tryptamine ($t(4) = 4.754$, $p = 0.009$), a monoamine alkaloid synthesised from the decarboxylation of the non-essential amino acid tryptophan via tryptophan decarboxylase (Mousseau, 1991). Tryptamine is a neuroactive molecule, which may function as a neuromodulator through indirect involvement in neurotransmission (Mousseau, 1993). Although there is limited data on the abundance and role of tryptamine in in vitro models, tryptamine has been detected in rat brain homogenate by liquid scintillation spectroscopy, LC and immunolabelling techniques (Saavedra and Axelrod, 1973; Dabadie and Geffard, 1993; Jiang et al., 2006). Using LC, Jiang et al. (2006) estimated the tryptamine concentration in the rat brain was 3.5 pM.g$^{-1}$ tissue. As tryptamine is synthesized from tryptophan, a decline in tryptamine may indicate a decline in abundance of the amino acid. However, tryptophan was not detected in the analysis of lupeol-treated or control samples, complicating the proposal of this conclusion. A decline in tryptamine may alternatively be an associated biochemical response to lupeol not previously identified, or indicate interactions of lupeol with neurotransmission, as was previously proposed due to fluctuations in GABA and tyrosine in Tables 3.2, 3.3 and 3.4.
3.5.3.2 Effect of Caffeine on Metabolite Profiles of B50 Cell Cultures

PCA was used to examine the metabolic effect of the neurotoxin caffeine on B50 cell culture metabolite profiles after 24 hours of incubation, relative to cell cultures incubated for the same time with a vehicle control. Scores plots of caffeine-treated and control cell and media samples are presented in Figure 3.15.

Figure 3.15 illustrates 87% of the variation between cell samples incubated for 24 hours can described by PC 1 and 2. This is greater than the 82% variance described by PCs modelling the media samples incubated for sample time period. Figure 3.15 demonstrates caffeine application to cell cultures induced changes in the metabolite profiles of both cells and media. The dissimilarities of profiles in caffeine-treated cultures relative to controls are best exemplified in cell samples, which are differentiated into two clusters by PC-1 (Figure 3.15A). Figure 3.15B also identifies clustering of treated and untreated groups separated by PC-2, again indicating general dissimilarity in metabolite profiles in response to caffeine.
Figure 3.15: PCA plots of B50 rat cortical neuronal cell culture samples exposed to 1 mM caffeine (n = 3) or vehicle control (n = 3) for 24 hours and analysed by GC-MS. (A) Scores plot of cell samples situated according to relative intersample similarity with identified groupings of replicates. (B) Scores plot of media samples situated according to relative intersample similarity with identified groupings of replicates.
Metabolites most influencing the intersample variation of the samples in Figure 3.15 were further examined and compared to determine those increasing or decreasing in lupeol-treated samples relative to controls, as illustrated in Table 3.5.

**Table 3.5:** Differences in metabolites determined by PCA to most influence the variance of samples from B50 rat cortical neuronal cell cultures exposed to 1 mM caffeine (n = 3), relative to samples exposed to vehicle control (n = 3), for 24 hours. Metabolites are presented by sample type and relative metabolite class, where “↑” indicates greater metabolite levels in caffeine-treated samples relative to vehicle control samples and “↓” indicates lower levels in caffeine-treated samples relative to controls.

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<td>Ribose</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
</tr>
<tr>
<td>Unidentified Carbohydrate ↓</td>
<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td><strong>Others</strong></td>
</tr>
<tr>
<td>GABA ♠</td>
<td>N-Acetylglutamic Acid ↓</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>Urea ↓</td>
</tr>
<tr>
<td>N-Acetylglutamic Acid</td>
<td></td>
</tr>
<tr>
<td>Phosphoric Acid</td>
<td></td>
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<tr>
<td>Pyroglutamic Acid</td>
<td></td>
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<tr>
<td>Tryptamine ●</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>↑</td>
</tr>
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</table>

♣ Statistically significant (t(4) = -4.187, p = 0.014); ♦ Statistically significant (t(4) = -5.192, p = 0.007); ♠ Statistically significant (t(4) = -3.936, p = 0.017); ● Statistically significant (t(4) = -4.267, p = 0.013); v Statistically significant (t(4) = 2.866, p = 0.090).
Caffeine is an adenosine A1 and A2 receptor antagonist that increases cholinergic activation and inhibits the hydrolysis of cyclic adenosine monophosphate (cAMP) into AMP, thereby increasing intracellular cAMP levels. cAMP is an intracellular secondary messenger responsible for regulation of numerous cellular enzymes and metabolic pathways including protein kinase A, glycogenolysis, lipolysis, transcription and membrane permeability (Pandey and Shukla, 2005). The biochemical consequences of caffeine administration are therefore varied but include increased catabolic activities (and consequential increases in ATP production), stimulation of CNS activity and thermogenesis (Kobayashi-Hattori et al., 2005; Kim et al. 2010). The metabolic impacts of caffeine have been previously explored in humans (Astrup et al., 1990; Tofovic et al., 2001; Kobayashi-Hattori et al., 2005). For example, Astrup et al. (1990) reported the administration of 100 – 400 mg of caffeine to humans significantly increased energy expenditure dose dependently and resulted in a positive thermogenic response correlated with plasma caffeine levels. Kobayashi-Hattori et al. (2005) determined rats fed 5 mg of caffeine showed significantly increased free fatty acids levels in serum when measured every 30 minutes over 3 hours following administrated. The increase in free fatty acids was also correlated with significant increases in dopamine, norepinephrine and epinephrine, suggesting the induced lipolysis may be mediated by catecholamines. However, there are conflicting data in the field on the impact of caffeine. For example, Tofovic et al. (2001) determined the consumption of a 0.1% caffeine solution in obese rats did not result in statistically significant differences in plasma triglycerides or glycerol levels relative to rats receiving a placebo solution. However, caffeine-treated rats did display significantly increased cholesterol levels after 4 and 8 weeks of chronic exposure.

The data in Table 3.5 support a number of observations made in previous reports investigating caffeine administration. For example, the reductions in carbohydrates suggest increased ATP production (potentially suggesting a thermogenic response) exemplified by a significant decline in fructose \((t(4)=2.866, p = 0.090)\). The reduction in glycerol-3-phosphate also supports an increase in fatty acid catabolism, as the metabolite is generated from the glycerol, the triose sugar backbone of triglycerides and glycerophospholipids, via glycerol kinase (Nelson and Cox, 2008). The increase in cholesterol reported in Table 3.5 is also consistent with result of Tofovic et al.
(2001), which identified the same consequence in rat plasma following oral administered of caffeine.

Table 3.5 also indicates amino acids were less abundant in caffeine-treated cells with significant declines in intracellular alanine ($t(4) = -4.187, p = 0.014$) and leucine ($t(4) = -5.192, p = 0.007$). Conversely, amino acids increased in extracellular abundance. Table 3.5 may therefore indicate an efflux or loss of amino acids from cells in response to caffeine, evidenced by the complementary changes in both serine and isoleucine, which decreased intracellularly whilst increasing extracellularly. Alternatively, since the amount of cells in culture determines the quantity of metabolites analysed (León et al., 2013), low abundances of intracellular amino acids may reflect observations from Figure 3.11 where cells were visually sparser and less abundant in culture relative to controls. However, subsequent cell concentration determination (Figure 3.12) indicated the observed declines in cell concentration were not statistically significant, a result that does not support the conclusion of reduced cell concentration accounting for declines in metabolite abundances. The decrease in amino acids may therefore reflect increased catabolism in response to caffeine. For example, alanine, glycine and serine may be catabolised to pyruvate and enter the citric acid cycle for ATP production, while other amino acids may also be converted to other citric acid intermediates such as leucine and isoleucine forming acetyl-CoA, valine generating succinyl-CoA, aspartate forming oxaloacetate and threonine being catabolised to acetyl-CoA, pyruvate or succinyl-CoA (Nelson and Cox, 2008).

There are also similar reports on the impact of caffeine on amino acid utilization. For example, Jordá et al. (1988) measured in vitro declines in intracellular leucine, isoleucine, tyrosine and phenylalanine 30 minutes after administration of 1 mM caffeine to rat hepatocytes. Wajda et al. (1989) found mice consuming caffeine for 3 weeks and demonstrating a caffeine concentration of $113 \pm 19 \mu g.g^{-1}$ in the cerebral cortex exhibited reduced glycine, alanine, serine and threonine, closely reflecting the results of this work. Although the in vitro method in this work examined neuronal cells over 24 hours, it may be expected that similar metabolic outcomes result from the same compound, thereby accounting for the declines in intracellular amino acids.
Table 3.5 also illustrates reduced carbohydrates in caffeine-treated cells. Similarly to the decline in abundance of amino acids, carbohydrates may be less abundant due to carbohydrate loss from cell membrane damage. Alternatively, the declines in citric acid cycle intermediates (succinate and malate) in response to caffeine treatment may also represent an increased utilisation of energy sources such as fructose and glucose, which also declined in abundance.

GABA was also significantly less abundant in caffeine-treated cells ($t(4) = -3.936, p = 0.017$) (Table 3.5). This is in agreement with reports investigating the metabolic impact of caffeine toxicity both *in vitro* and *in vivo* including the previously described work by Wajda *et al.* (1989), which found mice receiving chronic caffeine administration demonstrated reduced GABA. However, there is disagreement in the field on the mechanism through which caffeine decreases GABA, which may be due to caffeine-induced increases in Ca$^{2+}$ mobilisation, which depresses GABA$_A$ receptor response (Desaulles *et al.*, 1991). Alternatively, caffeine may have an inhibitory effect on GABA transmission, reducing current and restricting speed of onset in a mechanism unrelated to Ca$^{2+}$ mobilisation, as has been observed in *in vitro* studies of animal neuronal cells (Akopian *et al.*, 1998; Taketo *et al.*, 2004).

Intracellular tryptamine was also significantly reduced in caffeine-treated cells ($t(4) = -4.187, p = 0.013$) (Table 3.5), a result consistent with cells treated with lupeol only (data not presented). As there is an absence of reports examining the impact of caffeine on tryptamine, it could be proposed that caffeine toxicity inhibits the production or abundance of tryptamine via its effects on intracellular Ca$^{2+}$ mobilisation. Alternatively, as was proposed for the same observations of declines in GABA and tryptamine in lupeol-treated cells, the impact of caffeine may result in inhibition or modulation of neurotransmission or similar neuroactive pathways, indirectly impacting tryptamine abundance. As caffeine-treated cells displayed general reductions in amino acids, an unobserved reduction in tryptophan may also account for reductions in tryptamine.

Table 3.5 also reports a caffeine-induced decline in myo-inositol, a constituent of the intracellular secondary messenger inositol (1,4,5)-triphosphate (IP$_3$) and the minor structural component of eukaryotic cell membranes phosphatidylinositol 4,5-bisphosphate (Nelson and Cox, 2008).
Although Bezprozvanny et al. (1994) previously reported 1.64 mM caffeine induced a half inhibition of IP$_3$-gated Ca$^{2+}$ channels in canine cerebellum endoplasmic reticulum vesicles, there do not appear to be data indicating caffeine reduced myo-inositol or IP$_3$ abundance. The decline in myo-inositol may therefore be a unique observation of the toxicity of caffeine, resulting in a degradation of IP$_3$-gated channels or secondary messenger molecules, or a breakdown of inositol-containing cell membrane components.

Caffeine administration to B50 cell cultures therefore induced a number of significant changes in metabolites with a general trend in reductions of intracellular (and increases in extracellular) amino acids and carbohydrates. Intracellular tryptamine and GABA were also significantly reduced. These data therefore suggest caffeine induced a toxic response *in vitro* characterised by increased utilisation of substrates for ATP production, potentially including neuromodulation by inhibiting tryptamine and GABA production or utilisation. Alternatively, caffeine may have induced cell membrane damage resulting in metabolite efflux.

### 3.5.3.3 Effect of Co-Administration of Lupeol and Caffeine on Metabolite Profiles of B50 Cell Cultures

PCA was used to examine the metabolic effect of co-administration of caffeine with lupeol on B50 cell culture metabolite profiles after 24 hours, relative to cell cultures incubated for the same time with a vehicle control. Scores plots of caffeine-treated and control cell and media samples are presented in Figure 3.16.

Figure 3.16 illustrates that 80% of the variation between cell samples incubated for 24 hours can described by PC 1 and 2. This is greater than the 75% variance described by PCs modelling the media samples incubated for sample time period. From Figure 3.16, it is apparent that caffeine and lupeol application to cell cultures resulted in changes in the metabolite profiles of cells, as indicated by the separation of treatment groups in Figure 3.16A to a reproducible set of control samples to the right of the plot, from a broad, relatively unreproducible set of treated replicates to the left of the plot.
Figure 3.16: PCA plots of B50 rat cortical neuronal cell culture samples exposed to 10 µM lupeol with 1 mM caffeine (n = 3) or vehicle control (n = 3) for 24 hours and analysed by GC-MS. (A) Scores plot of cell samples situated according to relative intersample similarity with identified groupings of replicates. (B) Scores plot of media samples situated according to relative intersample similarity with identified groupings of replicates.
Media samples illustrated in Figure 3.16B, however, appear broadly dispersed across the plot with no defined separation between the treatment and control groups. Metabolite profiles of the media samples from both groups were therefore minimally affected by the co-administration of caffeine and lupeol.

Metabolites most influencing the intersample variation of the samples in Figure 3.16 were further examined and compared to determine those increasing or decreasing in lupeol-treated samples relative to controls, as illustrated in Table 3.6.

**Table 3.6:** Differences in metabolites determined by PCA to most influence the variance of samples from B50 rat cortical neuronal cell cultures exposed to 10 µM lupeol with 1 mM caffeine (n = 3), relative to samples exposed to a vehicle control (n = 3), for 24 hours. Metabolites are presented by sample type and metabolite class, where “↑” indicates greater metabolite levels in lupeol and caffeine-treated samples relative to vehicle control samples and “↓” indicates lower levels in lupeol and caffeine-treated samples relative to controls.

<table>
<thead>
<tr>
<th>Intracellular Samples</th>
<th>Extracellular Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td><strong>Amino Acids</strong></td>
</tr>
<tr>
<td>Aspartate ♦</td>
<td>↓</td>
</tr>
<tr>
<td>Threonine ↓</td>
<td>Leucine ↑</td>
</tr>
<tr>
<td>Valine ↓</td>
<td>Methionine ◊ ↑</td>
</tr>
<tr>
<td></td>
<td>Serine ♣ ↑</td>
</tr>
<tr>
<td></td>
<td>Threonine ↑</td>
</tr>
<tr>
<td></td>
<td>Valine ✪ ↑</td>
</tr>
<tr>
<td></td>
<td>Aspartate ↓</td>
</tr>
<tr>
<td><strong>Carbohydrates</strong></td>
<td><strong>Carbohydrates</strong></td>
</tr>
<tr>
<td>Unidentified Carbohydrate ↑</td>
<td>Fructose ↑</td>
</tr>
<tr>
<td></td>
<td>Erythronate ↓</td>
</tr>
<tr>
<td></td>
<td>Malate ♣ ↓</td>
</tr>
<tr>
<td></td>
<td>Ribitol ✤ ↓</td>
</tr>
<tr>
<td></td>
<td>Ribose ● ↓</td>
</tr>
<tr>
<td></td>
<td>2 Unidentified Carbohydrates ↓</td>
</tr>
<tr>
<td>Succinate</td>
<td>↓</td>
</tr>
<tr>
<td>Unidentified Carbohydrate ↓</td>
<td></td>
</tr>
<tr>
<td><strong>Fatty Acids</strong></td>
<td><strong>Others</strong></td>
</tr>
<tr>
<td>Palmitic Acid</td>
<td>↑</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td><strong>Others</strong></td>
</tr>
<tr>
<td>Benzoic Acid</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Urea ↓</td>
</tr>
<tr>
<td>Tryptamine ▲</td>
<td>↓</td>
</tr>
</tbody>
</table>

♦ Statistically significant ($t(4) = -3.865, p = 0.018$); ◊ Statistically significant ($t(4) = -3.348, p = 0.029$);
♣ Statistically significant ($t(4) = -9.700, p = 0.001$); ● Statistically significant ($t(4) = -5.637, p = 0.005$);
• Statistically significant ($t(4) = 4.120, p = 0.015$); ◊ Statistically significant ($t(4) = 3.596, p = 0.023$);
❖ Statistically significant ($t(4) = 6.250, p = 0.003$); ▲ Statistically significant ($t(4) = 3.319, p = 0.029$).
From Table 3.6, it is apparent that the trends in metabolites observed in caffeine-treated samples relative to controls (Table 3.5) are also present in samples treated with both lupeol and caffeine relative to controls. For example, Table 3.6 illustrates a decline in intracellular amino acid levels, exemplified by a significant decline in aspartate \((t(4) = -3.865, p = 0.018)\). Threonine and valine were also less abundant in cells treated with both lupeol and caffeine (Table 3.6), as was observed in Table 3.5 in response to caffeine alone. Extracellular amino acids generally increased, exemplified by significantly greater abundances of methionine \((t(4) = 3.596, p = 0.023)\), serine \((t(4) = 6.250, p = 0.003)\) and valine \((t(4) = 3.319, p = 0.029)\) in response to co-administration (Table 3.6). Serine was also observed to increase in response to caffeine alone, although the increase was not statistically significant. These similarities therefore indicate similar biochemical consequences resulting from the application of lupeol and caffeine, relative to caffeine alone.

Table 3.6 also illustrates multiple statistically significant reductions in intracellular carbohydrate abundances, with a general increase in extracellular carbohydrates (although carbohydrate changes in the media were no significantly different). Carbohydrates that were significantly less abundant in samples receiving the co-administration (malate \((t(4) = -3.348, p = 0.029)\), ribitol \((t(4) = -9.700, p = 0.001)\), ribose \((t(4) = -5.637, p = 0.005)\) were also reduced in cell samples treated with only caffeine (Table 3.5) but not to a statistically significant extent. This comparison again suggests similar biochemical consequences resulting from the application of lupeol and caffeine, relative to caffeine only.

Intracellular tryptamine was also significantly less abundant \((t(4) = 4.120, p = 0.015)\) in cells treated with co-administration relative to controls. This result was observed in response to both lupeol (data not presented) and caffeine (Table 3.5), therefore supporting the observation that a reduction in tryptamine would again be observed in co-administered samples. As previously suggested, the decline in tryptamine may be a function of induced modulation of neural activity through an unidentifiable mechanism or a reflection of reduced tryptophan levels.

The similarities in metabolite differences of samples treated with either caffeine or lupeol and caffeine suggests similar biochemical activities were occurring between the two samples sets. As
previously suggested, amino acids and carbohydrates may have been actively transported out of cells or lost due to cell membrane damage as a consequence of caffeine administration. Further, amino acids and carbohydrates may have contributed to increased ATP production and were utilised in excess in response to caffeine, resulting from increased cAMP levels and consequently, increased lipolysis and thermogenesis (Kobayashi-Hattori et al., 2005; Kim et al. 2010). As malate and succinate are both intermediates of the citric acid cycle, an increased activity of the cycle is a valid inference from Table 3.6, again suggesting caffeine increased ATP generation despite the presence of lupeol. The latter conclusion is supported by the absence of statistically significant differences in extracellular carbohydrates, suggesting intracellular carbohydrates declines were not due to exportation or loss.

Significantly reduced levels of ribitol ($t(4) = -9.700, p = 0.001$) and ribose ($t(4) = -5.637, p = 0.005$) are also exhibited in Table 3.6. This is the first identification of these carbohydrates being significantly reduced in this work and identifies a potential difference in the impact of co-administration relative to caffeine-only administration. Ribose is a 5-carbon monosaccharide existing in either a straight chain (aldehyde) or ring (β-furanose) conformation, the latter of which is an integral component of cellular nucleic acids (Nelson and Cox, 2008). Ribose rarely exists freely in the cell, as it is primarily converted to ribose-5-phosphate (R5P) by ribokinase, utilising ATP as a phosphate donor with a concomitant production of adenosine diphosphate. R5P is utilised in nucleotide biosynthesis (Park and Gupta, 2008). A decline in free ribose may therefore indicate an increase in R5P production and consequently, nucleotide biosynthesis, perhaps for nucleic acid repair in response to the toxicity (specifically apoptosis) induced by caffeine.

Ribitol is an alcohol of ribose and a component of riboflavin (vitamin B$_2$), which exists as the cofactor flavin adenine dinucleotide (FAD) when bound to ADP (Stoker, 2010). FAD is typically bound to dehydrogenase enzymes such as succinate dehydrogenase, which in the citric acid cycle, converts succinate to fumurate. This conversion generates two high-energy electrons, reducing FAD to a reduced form (FADH$_2$), before entering the electron transport chain for the generation of ATP (approximately 1.5 molecules of ATP per electron pair) (Nelson and Cox, 2008). As it
has previously been suggested, caffeine increases ATP production within the cell and the reduction in ribitol may indirectly represent another consequence of this upregulation.

The biochemical consequences of caffeine administration therefore appeared to have been replicated in samples treated with co-administration of caffeine and lupeol. The presence of lupeol therefore had minimal impact on the effects of caffeine and afforded no neuroprotection against caffeine toxicity. This is supported by the similarity in appearance of cultures treated with caffeine or caffeine and lupeol (Figure 3.11). However, the statistically significantly reductions in intracellular carbohydrates observed in response to co-administrated but not observed in cells treated with only caffeine may suggest caffeine-induced toxicity occurred to a greater extent in the presence of lupeol.

Due to the absence of significantly different metabolites between control cell cultures and cultures treated with lupeol only, results of a comparison of lupeol and caffeine-treated cell cultures, relative to cultures treated with lupeol only, are not presented. PCA of this comparison largely reflected the results presented in Figure 3.16 and Table 3.6, whereby the co-administration of lupeol and caffeine induced decreases in intracellular amino acids and carbohydrates with increases in extracellular amino acids and carbohydrates. These data would be expected given the similarity of lupeol-treated to control cell cultures.

**3.5.3.4 Effect of Co-Administration of Lupeol and Caffeine on Metabolite Profiles of B50 Cell Cultures Relative to Caffeine Administration**

PCA was utilised to examine the metabolic effect of co-administration of caffeine with lupeol on B50 cell cultures after 24 hours of incubation, relative to cell cultures incubated for the same time with caffeine only. Scores plots of cell and media samples treated with co-administration or only caffeine are presented in Figure 3.17.

Figure 3.17 illustrates that 86% of the variation between cell samples incubated for 24 hours can described by PC 1 and 2. This is greater than the 85% variance described by PCs modelling the media samples under the same conditions.
Figure 3.17: PCA plots of B50 rat cortical neuronal cell culture samples exposed to 1 mM caffeine (n = 3) or 10 µM lupeol with 1 mM caffeine (n = 3) for 24 hours and analysed by GC-MS. (A) Scores plot of cell samples situated according to relative intersample similarity with identified groupings of replicates. (B) Scores plot of media samples situated according to relative intersample similarity with identified groupings of replicates.
Figure 3.17A indicates cell samples treated with only caffeine were clearly differentiated from relatively un reproducible samples of cells treated with both caffeine and lupeol. Figure 3.17B indicates both treatment groups display similar extracellular metabolite profiles. Figure 3.17 therefore indicates co-administration largely resulted in similar biochemical consequences in the media of cell cultures relative to treatment with caffeine only. However, co-administration induced a somewhat different biochemical response in cell samples relative to treatment of cells with caffeine only, indicating the application of lupeol with caffeine altered the biochemical impact of the neurotoxin.

Metabolites most influencing the variation of samples in Figure 3.17 were further examined and compared to determine those increasing or decreasing in samples treated with both lupeol and caffeine relative to samples treated with only caffeine, as illustrated in Table 3.7.

Initial observations of Table 3.7 indicate few statistically significant differences in metabolites between treatment groups. Table 3.7 is consistent with the PCA presented in Figure 3.17 by identifying 3 significant differences in metabolites in cell samples (accounting for the variation in Figure 3.17A) while there were no significant differences in metabolites between media samples (accounting for the similarity in extracellular metabolite profiles in Figure 3.17B). Table 3.7 therefore supports the notion that the media of samples receiving both lupeol and caffeine are similar to samples receiving only caffeine. Table 3.7 indicates the amino acid efflux or utilisation that has consistently resulted from caffeine application irrespective of the presence of lupeol, was greater in cells receiving caffeine with lupeol relative to cells only receiving caffeine. In particular, Table 3.7 illustrates a significantly reduced abundance of intracellular aspartate ($t(4) = 4.836, p = 0.008$), relative to caffeine-only-treated cells. This result was also observed when comparing cells co-administered lupeol and caffeine to controls (data not presented), while a comparison of caffeine-only to controls also yielded a reduced level of aspartate (Table 3.5). These data suggest the addition of lupeol with caffeine particularly impacted upon aspartate. This may be a consequence of lupeol up-regulating processes that utilise aspartate such as the citric acid cycle, whereby aspartate is metabolised to oxaloacetate, a citric acid cycle intermediate.
Table 3.7: Differences in metabolites determined by PCA as most influential on the variance of samples from B50 rat cortical neuronal cell cultures exposed to 10 µM lupeol with 1 mM caffeine (n = 3), relative to samples exposed to 1 mM caffeine (n = 3), for 24 hours. Metabolites are presented by sample type and metabolite class, where “↑” indicates greater metabolite levels in lupeol and caffeine-treated samples relative to caffeine-treated samples, while “↓” indicates lower metabolite levels in lupeol and caffeine-treated samples relative to caffeine-treated samples.

<table>
<thead>
<tr>
<th>Intracellular Samples</th>
<th>Extracellular Samples</th>
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<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td><strong>Amino Acids</strong></td>
</tr>
<tr>
<td>Aspartate♦</td>
<td>↓</td>
</tr>
<tr>
<td>Threonine</td>
<td>↓</td>
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<tr>
<td>Valine</td>
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<td>Phenylalanine</td>
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<tr>
<td><strong>Carbohydrates</strong></td>
<td><strong>Carbohydrates</strong></td>
</tr>
<tr>
<td>Erythronate</td>
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<tr>
<td>Ribitol♣</td>
<td>↓</td>
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<tr>
<td>Ribose♠</td>
<td>↓</td>
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<tr>
<td>Succinate</td>
<td>↓</td>
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<tr>
<td>2 Unidentified Carbohydrates</td>
<td>↓</td>
</tr>
<tr>
<td>2 Unidentified Carbohydrates</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td><strong>Others</strong></td>
</tr>
<tr>
<td>Benzoic Acid</td>
<td>↑</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>↓</td>
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♦ Statistically significant ($t(4) = 4.836, p = 0.008$); ♣ Statistically significant ($t(4) = 9.511, p = 0.011$); ♠ Statistically significant ($t(4) = 3.633, p = 0.022$).

A significant reduction in aspartate may therefore indicate an increase in the conversion of oxaloacetate to citrate in an up-regulation of the cycle for ATP production (Nelson and Cox, 2008). This inference has been previously suggested to result from caffeine application and may therefore represent an established impact of caffeine increasing ATP as a function of thermogenesis or fatty acid metabolism, potentiated by lupeol.

Intracellular ribitol ($t(4) = 9.511, p = 0.011$) and ribose ($t(4) = 3.633, p = 0.022$) were also significantly reduced in co-administration treated samples relative to caffeine-treated samples, following a trend of decreased intracellular carbohydrates (Table 3.7). It was previously
suggested that a decrease in ribitol was a function of caffeine-induced increases in ATP production, as ribitol is a structural component of FAD - a cofactor bound to enzymes such as succinate dehydrogenase that facilitates electron transport and subsequent ATP production. A decline in ribose was similarly suggested to result from caffeine via increased nucleotide synthesis, perhaps to repair or replace nucleotides affected by caffeine toxicity. As similar decreases in ribitol and ribose are presented in Table 3.7, it could be argued that both of these processes are potentiated in the presence of lupeol due to an unknown mechanism. Additional intracellular carbohydrates decreasing in response to co-administration relative to caffeine only may further indicate a potentiation of the biochemical impact of caffeine.

Increases in extracellular metabolites presented in Table 3.7 largely echo existing observations of the metabolic impact of caffeine. However, the observation that these differences were greater in samples receiving co-administration relative to caffeine only again signifies that the addition of lupeol with the toxin altered the impact of caffeine by potentiating the induced biochemical changes. From Table 3.7 it is therefore evident that although the addition of lupeol with caffeine generally imparts few significant metabolic changes relative to caffeine only exposure, intracellular aspartate, ribitol and ribose are particularly affected by co-administration with lupeol, suggesting the triterpenoid may increase their utilisation through an undefined mechanism.
4. General Discussion & Conclusion

4.1 Instrumental Analysis of Lupin-Derived Lupeol

In a characterisation of lupin-derived lupeol, this work aimed to apply the QuEChERS approach to the extraction of lupeol from lupin seeds. Two sample sets of whole lupin seeds were extracted using two different QuEChERS extraction kits, with the resulting matrices analysed by GC-MS. In each replicate sample, lupeol was identified via mass spectral comparison to the NIST database, indicating a QuEChERS approach could be successfully applied to the extraction of lupeol from lupin seeds. This use of QuEChERS appears to be a previously unreported application, providing a novel means of sample preparation for lupeol and lupin analysis. Given that QuEChERS has previously demonstrated applications in the recovery of biologically significant residues from plant materials and in this work, resulted in the co-extraction of other bioactive compounds such as $\gamma$-tocopherol, QuEChERS may be useful in simultaneous determinations of pharmacologically beneficial and potentially toxic molecules (such as pesticide residues and the 3 lupin alkaloids co-extracted in this work). QuEChERS may therefore be used to provide a holistic view of the chemical constituents of biological matrices, reducing time and associated costs of analysis by extracting multiple, bioactive compounds with a single, quick, easy, cheap, effective, rugged and safe methodology, readily applicable to instrumental analysis.

However, these data are somewhat limited by the absence of optimised parameters for extraction and instrumental analysis. For example, various aspects of QuEChERS can be modified to increase the efficacy of compound recovery such as the extraction solvent of choice (acetonitrile, acetone or methanol may be used) or choice of acid within the solvent (acetic, hydrochloric or formic acid). The QuEChERS kit utilised can also be customised to analyses. For example, alternative clean-up kits contain graphitised carbon black, a sorbent utilised for the removal of plant pigments, or aminopropyl, a clean-up reagent with similar functionality to PSA but less likely to induce degradation of alkaline-sensitive analytes. Quantities of QuEChERS reagents are also variable depending upon the kit of choice (United Chemical Technologies, 2012). Instrumental parameters including volume of injection, type of injector, type of column, temperature of the column and ion source and method of ionisation could also be varied to
determine an optimised instrumental method to maximise analyte signal intensity. Further analyses of lupin-derived lupeol should therefore incorporate method optimisation into the experimental work to ensure maximum extraction efficacy and analyte response, thereby developing a method tailored to the analysis of lupeol in lupin matrix.

This experimental work also aimed to quantitatively determine the abundance of lupeol in whole lupin seeds and isolated seed fractions. This aim was not achieved due to time restrictions and confounding instrumental difficulties, representing a notable limitation to the results presented. However, a comparison of 3 abundant ions visible in the mass spectra of lupeol with an ISTD was performed to determine a single, most-reproducible ion that could be applied to quantitative analyses. The determination of ions for the identification of lupeol and the ISTD also informed a comparison of two QuEChERS extraction kits, with results signifying a kit housed in a 50 mL centrifuge tube provided more reproducible analyses than a kit with comparable constituents housed in a mylar pouch. This result is valuable to ongoing analyses of lupeol in order to ensure future reproducibility in analyses by identifying the limitation that different QuEChERS kits containing different quantitates of reagents can affect both the results and reproducibility of analysis. However, the generation of quantitative data should be a primary focus of future related experimental work, as such data would inform the impact of lupin consumption and establish pharmacological outcomes from lupeol administration. Quantitative data could be achieved through the use of GC with MS/MS by QQQ or QTOF systems as mass analysers, which are suited to trace analysis and the detection of compounds in complex matrices such as lupins. MS/MS would also permit the development of instrumental methods monitoring ions specific to lupeol for sensitive and selective determinations, an endeavour partially achieved through the identification of \( \text{m/z} \) 135.2 as the most reproducible of three ions tested in the mass spectra of lupeol.

A non-quantitative comparison of corrected lupeol peak areas in whole lupin seeds with isolated seed kernel, hull and germ, elucidated the potential localisation of lupeol to the seed hull, where lupeol was significantly more abundant than whole seeds or germ. Lupeol was also absent from
isolated seed kernels. The abundance of lupeol in *L. angustifolius* complements existing data on the occurrence of lupeol in taxonomic groups, supplementing existing reports describing the abundance of lupeol in various eukaryotic species and the *Lupinus* genus. To the knowledge of the author, this is the first report identifying the occurrence of lupeol in *L. angustifolius* and the potential localisation of lupeol to the hull of *L. angustifolius* seeds. Localisation of lupeol to the seed hull may indicate a functional role of the triterpenoid as a secondary metabolite expressed to protect the contained embryo against microorganism attack or infection. However, further work on the specific role of lupeol must be conducted to more appropriately test this notion.

The abundance of lupeol in lupin seed hulls is also of commercial significance. As a potential phytotherapeutic agent, lupeol isolation could be maximised by separation of the seed coat from the remaining seed using dehulling mechanisms. Research sectors investigating lupeol may also benefit from dehulling prior to extraction, thereby maximising lupeol recovery and reducing time and costs associated with the extraction of lupin-derived lupeol from whole seed. Consumers could also gain greater therapeutic and bioprotective benefits from lupeol by replacing existing lupin kernel or whole seed meals utilised in lupin-containing pasta, tofu, breads and beverages, with milled lupeol hull. While increasing consumption of isolated hulls may increase lupeol consumption and therefore provide for greater pharmacological benefits, a more thorough comprehension of the bioavailability of lupeol from lupin hulls would need to be gained prior to the marketing of these ideals. Additional commercial opportunities are possible through the use of plant-breeding strategies to selectively generate lines exhibiting increased seed coat thickness, which may generate seeds expressing greater quantities of lupeol. Although this aim may be possible given that environmental conditions impact significantly upon seed coat thickness, a greater understanding of lupin seed coat biology and the synthesis of lupeol within the three cell layers composing the seed coat would first need to be explored (Miao *et al.*, 2001).

In addition to the commercial significant of this work, replicate analyses should be conducted to validate the data provided in this report using a larger number of replicate seed fraction samples and repeating the protocols utilised. An analysis of lupin-derived lupeol using *L. angustifolius*
seeds derived from other locations would also be of value to signify whether the results were simply representative of the sample of lupin seeds provided or in fact true of the species regardless of the specific conditions under which the sample was cultivated. In addition, future studies could endeavour to determine the abundance of lupeol in other dietary lupins including *L. mutabilis* and *L. luteus* to further investigate the pharmacological value of members of the genus.

### 4.2 Metabolomics Analysis of the Effect of Lupeol on B50 Cell Cultures

Using a metabolomics approach, this worked aimed to investigate the impact of lupeol on metabolite profiles of B50 rat cortical neuronal cells. The impact of lupeol was investigated by determining cell concentration in culture and performing metabolomics analysis of cultures treated with 10 µM lupeol for 4 or 24 hours, relative to cultures treated with vehicle control.

Cell concentration determinations indicated there were no significant differences in the mean number of cells present per well after 4 or 24 hours, suggesting that at 10 µM, lupeol did not induce a toxic response *in vitro*. However, it could not be determined whether lupeol induced a therapeutic response. Lupeol exerted a generally negligible impact on cell concentration, however this should be considered with respect to the limitations of the experimental method. The process of determining cell concentration was subjective in nature, identifying the presence of cells as a measure of biological significance and thereby not accounting for the presence of damaged cells, those undergoing apoptosis or erroneous counts made by the analyst (Manthorpe, 2001). Future investigations on the impact of lupeol on cell cultures should therefore incorporate methodologies more suited to determining cell viability, perhaps by evaluating cytotoxicity or biochemical activity. Such approaches may include dye exclusion assays such as the trypan blue assay, a more rapid technique which utilises a high molecular weight dye that dead cells or those late in apoptotic stages incorporate and thus appear blue, readily distinguishing them from white (living) cells that exclude the dye (Puranam and Boustany, 1999). Methods based on biochemical function may also be utilised, such as the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, an established rapid and quantitative approach measuring live cells that perform a
In a metabolomics analysis, PCA of treated and control cell cultures after 4 hours suggested the culture media differed in response to lupeol while cells did not. A comparison of metabolites between treated and control samples yielded no statistically significant differences in metabolite peak areas in response to lupeol. Of the observed differences, mixed changes in the abundances of amino acids, carbohydrates and fatty acids were found. These changes included a reduction in tyrosine and an increase in GABA, potentially indicating an up-regulation of neurotransmitter synthesis, while arginine and urea decreased in abundance, suggesting an inhibition of the urea cycle. After 24 hours, treatment groups displayed somewhat dissimilar metabolite profiles of cells while media metabolite profiles were more similar. Again, no metabolites were significantly different between groups and there were no clear trends in amino acid or carbohydrate abundances in response to lupeol. Data therefore suggested a dynamic response in metabolite influx, efflux or utilisation in biochemical pathways such as the citric acid cycle for ATP generation in response to lupeol. However, tyrosine and GABA were again respectively decreased and increased in response to lupeol, indicting a sustained increase in neurotransmitter synthesis. When comparing lupeol-treated samples incubated for 4 or 24 hours, media metabolite profiles were readily differentiated by incubation time whilst cell samples were comparatively more similar. Although intracellular citrate significantly decreased over time, no other metabolites were significantly altered, with differences in control samples monitored at the same intervals largely reflecting the observed changes in cultures treated with lupeol, indicating both groups behaved similarly over time.

It could therefore be concluded that lupeol was either transported into the cell and rapidly metabolised, where it had minimal impact on metabolite transport and utilisation, or degraded over time in the media. This was evidenced by media samples exposed to lupeol exhibiting metabolic differences 4 hours after treatment, potentially due to the extracellular presence of
lupeol. However, those differences were normalised over time, as extracellular samples became more similar to controls after 24 hours, whilst cells became less similar to controls.

The lack of statistically significant differences in metabolites impacted by lupeol after 4 or 24 hours therefore suggested the compound resulted in a generally negligible impact on B50 cell culture biochemistry. This conclusion represents a significant limitation of the data and the analytical methods utilised, suggesting comparative approaches such as proteomics or transcriptomics may be more useful in elucidating the impact of lupeol by informing both genotypic and phenotypic changes. Such impacts could not be clearly determined in this work due to the nature of the methodologies and instrumentation employed, which prevented the analysis of many inorganic molecules, molecules with large m/z beyond the detection capabilities of GC-MS (such as nucleic acids) and compounds not applicable to derivatisation or otherwise not sufficiently thermally labile for GC-MS analysis. However, if further metabolomics approaches are to be utilised to investigate the effects of lupeol, a number of alternative instrumental platforms could be used, including the previously described NMR or LC-MS techniques, the latter of which is applicable to alternative mass analysers such TOF or hybrid methods of analysis such as QTOF (León et al., 2013).

The lack of conclusive data derived from the experimental work also highlights other limitations of metabolomics approaches. In this work, an untargeted approach was adopted to analyse metabolite profiles of samples in order to maximise the scope of analysis and provide the most comprehensive assessment possible of metabolic response to stimuli in a single analysis. Although this approach offers advantages in the determination of biochemical status and physiological response, the data were not quantitative and therefore unable to provide more specific metabolic impacts of lupeol. Raw peak area data, as was utilised in this investigation due ISTD RDSs being greater than 50%, may also be impacted by inter-sample instrumental variations or matrix effects such as ion enhancement or ion suppression, which impact upon signal intensities of detected metabolites and therefore affect determinations of peak area. Particularly abundant metabolites may also overload the GC and elute broadly over time,
interfering with signals of co-eluting compounds and preventing their determination, or distorting
analyses of compounds with similar RTs. To some extent, these detriments can be circumvented
by targeted approaches, which endeavor to provide absolute quantification and identification of a
single or small number of metabolites, potentially through MS/MS analyses of specific ions and
ion fragmentations (Hübschmann, 2001; Hsu and Drinkwater, 2001). Although such an approach
was difficult in this work due to the limited data on the metabolic impacts of lupeol, future
analyses may endeavor to achieve such an analysis by focusing on particular biochemical
pathways or biochemical markers of therapeutic activity.

Additional limitations of the results are observed in the presentation of several unknown
carbohydrates that were altered by the addition of compounds to cell cultures, which despite the
availability of mass spectra, could not be identified. Current instrumental approaches are also
limited in the number of metabolites that may be potentially detected, meaning they cannot
provide a complete analysis of the metabolome of interest. Such limitations largely represent
restrictions not uncommon to metabolomics but may again be circumvented or alleviated by
utilising multiple instrumental platforms, optimising instrumental parameters or the use of a
greater number of internal standards for peak identification.

The small sample size of 3 replicates per treatment group also limits the conclusions drawn from
the data, necessitating the acknowledgement that large variations in a single replicate may have
skewed the mean data generated and thus distorted the formulation of accurate conclusions
(Čuperlović-Culf et al., 2010).

The use of an *in vitro* model to determine and interpret biological consequences should also be
acknowledged, given that physiological response and therefore metabolic activities can be more
dynamic and complex in multicellular organisms. However, mammalian *in vitro* models, such as
the B50 cell line utilised in this work, provide useful representations of biological systems that
can be readily manipulated with controlled variables to allow the generation of relevant and
meaningful biological conclusions (León et al., 2012). The cell line utilised in this work is an
established model in the field and has been previously used to investigate neurotransmitter
synthesis, neuronal cell behaviour and neurotoxicity (Bottone et al., 2008; Green, 2012; Ibegbu et al., 2012). The work performed in this study therefore contributes to the existing knowledge of B50 cell culture behaviour and supports the ongoing utilisation of the cell line for in vitro investigations of neurotoxicity as a useful and representative model.

In addition, the experimental design did not encompass a broad range of concentrations of lupeol administration. Although time constraints did not permit a wider examination of the impact of varying the concentration of lupeol, future work should endeavor to investigate this using neuronal cell cultures and metabolomics approaches to ensure hypotheses on the metabolic impact of lupeol are appropriately tested. Such work could generate a dose-response curve examining the impact of lupeol on cell viability and/or metabolites such as citrate, which was significantly altered over time in response to lupeol. Similarly, a broader range of exposure times should be tested with lupeol, particularly given that some metabolites exhibit rapid turnover rates, which may be less than 1 mM per second (Villas-Bôas, 2007). Future examinations could therefore employ metabolomics analyses of cultures at shorter time periods such as 30 minutes or 1 hour post-exposure.

4.3 Metabolomics Analysis of Lupeol as a Potential Neuroprotectant

This worked also aimed to investigate the activity of lupeol as a potential neuroprotectant against a neurotoxic compound. This investigation was conducted by visual observation, determinations of cell concentration and metabolomics analysis of B50 cell cultures treated with 10 µM lupeol, 1 mM caffeine, co-administration of 10 µM lupeol with 1 mM caffeine or vehicle control.

Cell imagery of each group 24 hours after treatment suggested lupeol had a negligible impact on cell culture appearance. However, caffeine treatment induced cellular aggregation and a decline in cell concentration, potentially due to apoptosis. Cultures treated with lupeol and caffeine appeared similar to caffeine-treated cultures, displaying losses of cell numbers and increased cellular aggregation.
Cell concentration determinations again concluded lupeol had no significant impact on the number of cells present after 24 hours. Treatment with caffeine or lupeol with caffeine also failed to result in a statistically significant difference in cell concentration. However, raw data suggested lupeol marginally increased cell concentration whilst caffeine reduced cell concentration. The reduction in concentration was greatest when caffeine was co-administered with lupeol, again suggesting lupeol afforded no protection against caffeine toxicity and may have resulted in a potentiation of toxicity.

Metabolomics analysis suggested lupeol again had minimal impact of the metabolite profiles of cell cultures, with the exception of a significant decline in intracellular tryptamine, potentially indicating an impact on neural activity. Caffeine application generally reduced intracellular amino acids and carbohydrates, with significant declines in intracellular alanine and leucine, suggesting increased catabolism. Intracellular GABA and tryptamine were also significantly reduced by caffeine administration, indicating a neuromodulatory effect or inhibitory effect on GABA synthesis, potentially through inhibition of intracellular Ca\(^{2+}\) signalling. Extracellular amino acids and carbohydrates also increased, exemplified by a significant increase in extracellular fructose, suggesting a loss or efflux of intracellular molecules due to apoptosis or an up-regulated exportation of compounds mediated by caffeine.

Co-administration of lupeol and caffeine induced similar trends in metabolite differences with reduced intracellular and increased extracellular amino acids and carbohydrates relative to controls. However, the number of significantly different metabolites observed in samples treated with co-administration, was greater than the number of metabolites significantly different in caffeine-treated samples relative to controls, suggesting the addition of lupeol altered the impact of caffeine and potentially increased the toxicity of the neurotoxin. Differences in metabolites in response to co-administration included significantly reduced intracellular aspartate, malate, ribitol and ribose, with significantly increased extracellular methionine, serine and valine. Again, these differences largely indicated a loss or increased utilisation of substrates. However, the result that
many of these differences were not statistically significant in response to caffeine alone, further suggested lupeol increased caffeine-induced intracellular metabolite loss or utilisation.

A comparison of co-administration treated cultures to caffeine-only treated cultures reinforced the notion that lupeol altered or increased the toxicity of the alkaloid, as co-administration resulted in greater reductions in aspartate, ribitol and ribose relative to caffeine treatment. However, extracellular metabolites were not significantly different indicating lupeol principally impacted intracellular metabolism.

Lupeol therefore resulted in minimal impact on intracellular or extracellular metabolites in isolation, suggesting the compound induced no in vitro toxicity at a dose of 10 μM to B50 cell cultures. At 1 mM, caffeine induced a toxic response, characterised by declines in intracellular amino acids and carbohydrates, whilst simultaneously increasing extracellular amino acids and carbohydrates. Metabolite differences suggested caffeine neurotoxicity induced up-regulated carbohydrate, amino acid and fatty acid catabolism for increased ATP production and thermogenesis and/or increased metabolite efflux from the cell due to cell membrane damage or stimulated exportation mechanisms. In combination with lupeol, aspartate, ribitol and ribose further decreased intracellularly suggesting an altered toxicity profile or potentiated toxicity of caffeine. Lupeol therefore exhibited no neuroprotective properties at a dose of 10 μM against a 1 mM acute dose of caffeine and further, may have potentiated or in some way altered the metabolic impact of caffeine.

The investigation of lupeol as a potential neuroprotectant largely presents similar limitations to those previously described, including the detriments of estimating cell concentration and the untargeted metabolomics approach utilised, the capabilities of GC-MS, use of small sample sizes and the nature of in vitro models. However, the identification of clear trends in amino acids and carbohydrates with several significant differences in metabolites in response to caffeine and co-administration of caffeine with lupeol does suggest this approach is somewhat useful in the identification of in vitro neurotoxicity. Further, these data indicate future in vitro experiments could be conducted utilising the B50 cell line, particularly in metabolomics analyses of
neurotoxicity. This work could therefore be used as a foundation to more closely investigate the impact of caffeine on amino acid and carbohydrate metabolism through quantitative (targeted) analysis in a potential elucidation of the toxicodynamics of caffeine or other neurotoxins.

As previously described, future related analyses may benefit from alternative approaches to cell assessment such as viability determinations to avoid the subjective nature of cell counting. In addition to approaches previously discussed, the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay has been applied to analyses of lupeol, detecting changes in nuclear DNA fragmentation during apoptosis (Lee et al., 2007c; Saleem et al., 2008; Kumari and Kakkar, 2012). Given the mode of toxicity induced by caffeine, the latter assay may be particularly applicable to future work investigating neuroprotection.

Further research endeavouring to examine possible neuroprotective properties of lupeol should also explore a variety of concentrations of lupeol and the neurotoxin to determine if a greater dose of lupeol influences caffeine toxicity. Conversely, the capacity of lupeol to afford neuroprotection against reduced doses of caffeine could also be explored. Further, lupeol could be investigated as a potential neuroprophylactic agent by administering the compound prior to neurotoxin application, particularly given that some reports have demonstrated lupeol is an effective therapeutic agent when administered prior to the induction of disease or the administration of a pathogen. Chronic exposure to lupeol could also be explored, particularly given that lupeol is effective against inflammation or cancers when administered in vitro for multiple days in a row.

4.4 Conclusion

This work achieved both a characterisation of lupeol in *L. angustifolius* seeds through instrumental analysis and a metabolomics analysis of the impact of lupeol alone and as a potential neuroprotectant against caffeine toxicity *in vitro*. The QuEChERS approach was successfully applied to the extraction of lupeol from whole lupin seed, isolated hull and germ, with comparisons made by GC-MS QQQ indicating lupeol was significantly more abundant and potentially localised in the hull of lupin seeds whilst absent from isolated kernels. Results
therefore suggested a novel application of the QuEChERS approach and additionally, suggested dehulling protocols could be utilised to maximise the extraction of lupin-derived lupeol in related research-based or industrial sectors. This work also indicated the effective application of sample preparation methods and GC-MS to an exploration of the endo- and exometabolome of B50 rat cortical neuronal cells. An insight into the metabolic impacts of lupeol as a therapeutic agent and caffeine as a neurotoxin was also provided, indicating lupeol had a negligible direct impact on cell cultures, while caffeine and co-administration of caffeine with lupeol induced a visually toxic response with reduced intracellular amino acids and carbohydrates and increased extracellular amino acids and carbohydrates. Potential neuroprotective properties of lupeol against caffeine toxicity were evaluated and proposed to be ineffective at the acute doses utilised, with results suggesting lupeol may have potentiated or in someway altered the metabolic impacts of caffeine. These data therefore informed the application of metabolomics to neurotoxicology and presented many additional opportunities for further in vitro investigations of the metabolic impacts of lupeol and caffeine, or the ability of lupeol to alter the toxicity of caffeine or other neurotoxins.
5. References


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