This article is downloaded from

[Image]

http://researchoutput.csu.edu.au

It is the paper published as:

**Author:** M. Kendall, M. Batterham, D. L. Callahan, D. Jardine, P. D. Prenzler, K. Robards and D. Ryan

**Title:** Randomized controlled study of the urinary excretion of biophenols following acute and chronic intake of olive leaf supplements.

**Journal:** Food Chemistry
**ISSN:** 0308-8146

**Year:** 2012
**Volume:** 130
**Issue:** 3
**Pages:** 651-659

**Abstract:** Olive leaf supplement was characterised by HPLC and administered to healthy young adults over 28 d (three tablets or equivalent liquid dose per day), or in a single bolus dose of three tablets (or liquid equivalent). Oleuropein was the major biphenol in the extracts. There are no data on the excretion of urinary end-products of the metabolism of the olive leaf biophenols. Following both chronic and acute ingestion neither oleuropein, nor its hydrolysis product, hydroxytyrosol, were detected in urine samples. However, glucuronic acid conjugates, derived from oleuropein aglycone were detected in all urine samples up to 6 h following acute ingestion. The data suggest that oleuropein is bioavailable, which is a necessary pre-condition for bioactivity.

**URLs:** http://researchoutput.csu.edu.au/R/-?func=dbin-jump-full&object_id=30311&local_base=GEN01-CSU01; http://dx.doi.org/10.1016/j.foodchem.2011.07.101

**Author Address:** pprenzler@csu.edu.au
krobrads@csu.edu.au
dryan@csu.edu.au

**CRO Number:** 30311
Randomized controlled study of the urinary excretion of biophenols following acute and chronic intake of olive leaf supplements

Megan Kendall¹, Marijka Batterham², Damien L. Callahan³, Daniel Jardine⁴, Paul D. Prenzler¹, Kevin Robards¹ and Danielle Ryan¹

¹ Charles Sturt University, EH Graham Centre for Agricultural Innovation, School of Agricultural and Wine Sciences, Wagga Wagga 2678, Australia

² Centre for Statistical and Survey Methodology, University of Wollongong, Northfields Ave Wollongong 2522, Australia

³ Metabolomics Australia, School of Botany, The University of Melbourne, Victoria 3010, Australia

⁴ Flinders Analytical, Faculty of Science & Engineering, Flinders University, Adelaide SA 5001

Corresponding author
Dr Danielle Ryan
Charles Sturt University
School of Wine and Food Sciences
Locked Bag 588
Wagga Wagga 2678
AUSTRALIA
Phone +61-2-6933 4382
Fax +61-2-6933 2866
Email dryan@csu.edu.au
Abstract
Olive leaf supplement was characterised by HPLC and administered to healthy young adults over 28 d (3 tablets or equivalent liquid dose per day) or in a single bolus dose of 3 tablets (or liquid equivalent). Oleuropein was the major biophenol in the extracts. There are no data on the excretion of urinary end-products of the metabolism of the olive leaf biophenols. Following both chronic and acute ingestion neither oleuropein nor its hydrolysis product, hydroxytyrosol, were detected in urine samples. However, glucuronic acid conjugates derived from oleuropein aglycone were detected in all urine samples up to 6 h following acute ingestion. The data suggest that oleuropein is bioavailable, which is a necessary pre-condition for bioactivity.

Keywords: Adults; excretion; healthy; oleuropein; urine

1. Introduction
As the world becomes more diet conscious, there is an increasing interest in olive products. Olive oil has been consumed over many centuries and health benefits have been associated with its intake. It has been studied extensively in recent times and its bioactivity (separate from its favourable fatty acid profile) has generally been attributed to an antioxidant effect by the phenolic fraction including oleuropein derivatives and hydroxytyrosol (Tripoli et al., 2005). Bioavailability, which is generally a pre-requisite for bioactivity, is a function of the capacity to be absorbed by the body and reach systemic circulation (Scalbert, Manach, Morand, Remesy, & Jimenez, 2005). An exception to this requirement is a direct antioxidant (or other) effect in the gastrointestinal tract, as this is independent of any phenolic absorption.
The rapid absorption of phenolic compounds in a dose dependent manner following consumption of olive oil has been demonstrated (Visioli et al., 2000). Data on the metabolism and excretion of olive biophenols are limited to olive oil as the vehicle. Some of the earlier data are contradictory and confusing (Edgecombe, Stretch, & Hayball, 2000; Del Boccio et al., 2003; Ranalli et al., 2006; Tuck & Hayball, 2002; Vissers, Zock, Roodenburg, Leenen, & Katan, 2002; Corona et al., 2006). A possible interpretation is that oleuropein is degraded by colonic microflora to species including hydroxytyrosol with the latter being well absorbed in the intestine (Corona et al., 2006). Moreover, the unavailability of standards has meant that a number of studies incorporated hydrolysis steps in the analytical methodology and thus did not distinguish between free phenols and their conjugated metabolites. Other studies demonstrated that the phenols are extensively metabolised in the gut and liver and are thus excreted mainly as Phase II metabolites of hydroxytyrosol and tyrosol (Miro-Casas et al., 2003a; Miro-Casas et al., 2003b; Miro-Casas et al., 2001). Two recent studies (Garcia-Villalba et al., 2010; Khymenets et al., 2011) have confirmed these earlier findings and provided quantitative data. However, the source of these metabolites remains unclear in some instances. Answers to such questions are not facilitated because in some instances the phenolic profile of the administered oils was not characterised and in others it showed several phenols (Garcia-Villalba et al., 2010; Khymenets et al., 2011) but with limited or no oleuropein.

Olive leaf contains many of the same phenols as the oil but in much higher concentrations (Silva, Gomes, Leitao, Coelho, & Boas, 2006). However, limited data suggest that the absorption of phenols is dependent on the vehicle of administration (Visioli et al., 2003) and yet there has been very limited in vivo research conducted on
these materials. We recently reported (Kendall et al., 2009) on the oxidative status of a healthy young population following ingestion of olive leaf supplements. A range of biomarkers did not show any change in the oxidative status of the cohort following supplement consumption. It is unclear whether the lack of bioactivity in our earlier study (Kendall et al., 2009) was a result of the population studied, poor bioavailability of the olive leaf phenols or poor choice of biomarkers. Leaf extracts are distinguished not only by higher phenolic concentrations than in olive oil but also by the phenolic profile (vide infra). Before repeating the oxidative status trial on a compromised group such as those with metabolic syndrome or a chronic disease such as diabetes we wished to gather more information on the metabolism and excretion of the olive leaf biophenols. This paper reports on the excretion and metabolism of oleuropein following both chronic and acute ingestion of olive leaf extract supplements.

2. Materials and Methods

2.1 Reagents and olive leaf supplement

Reagents and standards were obtained as previously reported (Ryan et al., 2002). Olive leaf supplements were provided as a capsule (Blooms Health Products ‘Olive Leaf’ batch 336163 70284) and a liquid extract (‘Olive Leaf Extract – Natural Flavour’ batch 731X-6A; Olive Leaf Australia, Coominya, QLD, Australia).

2.2 Human subjects

This study was approved by the Ethics in Human Research Committee of Charles Sturt University. Subjects (23 female, 22 male) were recruited from a class of first year health science students from Charles Sturt University, Wagga Wagga (Australia). Participants were generally in good health and exclusion criteria included being pregnant or breast-feeding, having any major chronic disease, wearing a pacemaker,
smoking cigarettes regularly, or having food allergies/intolerances. The average age, weight and body mass index (BMI) of the participants was 20.2 ± 3.0 y, 70.5 ± 16.8 kg and 23.2 ± 4.4 kg/m², respectively. Participants were instructed to consume one supplement (one capsule, or one 5 mL measure of liquid) three times per day, for 28 d as previously described (Kendall et al., 2009). A spot urine sample was supplied by each subject both at baseline and follow-up in sterile 70 mL containers. Collection time was not controlled. These samples were transferred to the laboratory within 30 min of voiding, and stored in a freezer at -20° C until analysis by HPLC and LC-MS.

For acute ingestion, four subjects (2 male, 2 female) orally ingested three olive leaf extract capsules, and baseline urine samples were collected, as well as spot samples up to 24 h post-ingestion. All urine excreted over the 24 h period was collected in sterilised collection jars and the volume measured. After a 24 h wash out period, the subjects consumed 15 mL of the liquid olive leaf extract and urine samples were collected as before. The procedure was replicated five times over two years.

To investigate the role of colonic microflora, rectal administration of the capsule extract was performed (in triplicate) on two healthy adult subjects (1 male, 1 female who had not presented with any illness in the previous 12 months OR who had not been treated with antibiotics in the preceding 12 months) by insertion as a suppository approximately 6-7 cm into the rectum. Correct positioning of the capsule was verified by a Registered Nurse who also confirmed that the capsule had melted after 15 min by digital examination. The inserted sample was retained for a minimum of 4 h. Urine samples were collected and analysed as before.
2.3 High performance liquid chromatography

HPLC was performed on a Varian 9021 solvent delivery system equipped with a Varian 9065 Polychrom UV diode array detector (190-367 nm). The system was maintained in a controlled temperature room at 21 ± 1 °C. A flow rate of 0.6 mL/min and an injection volume of 20 µL were used. Separation was performed by gradient elution on a 100 mm x 4.6 mm i.d., 3 µm, Polaris C-18 column (Varian, Australia) attached to a SecurityGuard guard cartridge (Phenomenex, Australia). Software used for data processing was Star Polychrom version 5.2. The mobile phases were freshly prepared and degassed under vacuum using Phenomenex Nylon 45 µm membranes and sonicated in a Sanophon ultrasonic bath (Ultrasonic Industries Pty. Ltd., Sydney, Australia) for 15 min prior to HPLC analysis.

Solvent A was a mixture of water: formic acid (100:1 v/v) and Solvent B was a mixture of acetonitrile: water: formic acid (50:50:1 v/v/v). A multi-step linear gradient analysis was used as follows: initial, 100% solvent A isocratic for 17 min; reducing to 96% at 20 min then isocratic to 25 min; reducing to 85% at 30 min and to 75% at 40 min then isocratic to 45 min; reducing to 65% at 50 min, to 30% at 55 min and then to 0% solvent A at 65 min. The system was equilibrated between runs for 20 min using the starting mobile phase composition.

Urine samples were analysed after filtration through a 0.45 µm filter (Advantec MFS, Inc., Dublin, CA, USA) with a sterile syringe. No other pre-treatment of samples was performed.

2.4 Liquid Chromatography-Mass Spectrometry
2.4.1 LC-MS

Preliminary analyses were performed on a Micromass Quattro micro tandem quadruple mass spectrometer (Waters, Manchester, UK) using electrospray ionisation (ESI). LC separation was provided by a Waters liquid chromatograph (Milford, USA), consisting of a 2695 Separation Module and 2487 dual wavelength UV detector operated at 240 and 280 nm. An SGE Wakosil C18 column (150 mm x 2 mm; 5µm) was used. Solvents and gradient program were as used for HPLC. An injection volume of 10 µL and a constant flow of 0.2 mL/min were used for each analysis. The entire flow from the LC was directed into the mass spectrometer. Data were acquired by the Masslynx data system for both the MS and UV data. The mass spectral data were acquired for four alternative scans; Scan 1: Positive ion mode, cone voltage 35 V; Scan 2: Positive ion mode, cone voltage 70 V; Scan 3: Negative ion mode, cone voltage 30 V; Scan 4: Negative ion mode, cone voltage 70 V. All scans were performed in the range 80 to 1500 m/z in 1 sec.

2.4.2 LC-QTOFMS

Samples were analysed by reversed phase liquid chromatography (LC; Agilent 1200) with QTOFMS (quadropole time-of-flight mass spectrometry; Agilent 6520) detection in both positive and negative ion modes. Separation was achieved using an Agilent Zorbax XDB C18 column (100 mm x 2.1 mm i.d.; 1.8 µm) and the column temperature was 35 °C. Solvent A was formic acid in water (0.1%) and Solvent B was formic acid in acetonitrile (0.1 %). The gradient commenced at 2 % B increasing to 30 % B at 5.0 min and 100 % B at 10.0 min. The gradient was held at 100 % B for 2 mins before returning to 2 % B at 12.1 mins (held at 2 % B until 17 mins). A flow rate of 0.4 mL/min was used together with an injection volume of 5 µL.
Conditions for the ESI were: nebuliser pressure 40 psi; gas flow-rate 8 L/min; gas temperature 300 °C; capillary voltage 4000 V; fragmentor voltage 140 V and skimmer voltage 65 V. Collision induced dissociation (CID) in the negative ion mode was achieved using a collision energy of 20 V. A scan rate of 2 scans/s was used for both full scan and MS/MS analyses, and data was collected in the range of 100–1600 m/z. The mass accuracy of the QTOFMS is ± < 2 ppm.

3. Results

3.1 Characterisation of olive supplements

Characterisation of the capsule and liquid supplements using HPLC and LC-MS (Kendall, Batterham, Prenzler, Ryan, & Robards, 2008) showed a single major phenolic peak that was identified from retention and mass spectral data (retention time of 61.1 min, m/z 541 ESI+, m/z 539 ESI−) as oleuropein by comparison to a commercial standard. The oleuropein content of the capsule and liquid was calculated as 20 mg/tablet and 22 mg/5 mL, respectively, via external calibration using an oleuropein standard. The liquid supplement was a much more heterogenous mixture when compared to the capsule supplement as evidenced by more peaks in the chromatograms (data not shown). A much smaller peak at 37.0 min in both capsule and liquid supplements was tentatively identified as hydroxytyrosol glucoside, based on previous work on olives (Ryan et al., 2002; Ryan, Robards, & Lavee, 1999) and olive waste (Obied et al., 2005).

3.2 Liquid chromatography profiles of urine samples

3.2.1 Profiles following chronic ingestion
HPLC profiling of the study participant’s urine samples at both baseline and following 28 d consumption was performed with photodiode array detection (PDA) monitoring at 220 and 280 nm. Selected chromatograms are shown in Figure 1. The chromatograms at 220 nm were dominated by a relatively small number of peaks with high intensity in contrast to the chromatograms generated at 280 nm which generally contained many peaks of comparable intensity. The major peaks in the 220 nm chromatograms included creatinine, uric acid and hippuric acid plus much lower concentrations of the 3- and 4-hydroxyhippuric acids. LC-MS plus use of commercial standards confirmed the presence of these compounds. Uric acid and creatinine were present in every sample and there was a semi-quantitative correlation with creatinine results from an assay kit (Kendall et al., 2009). Hippuric acid was detected in approximately 50% of urine samples with 1000-fold variation in concentration. This probably indicates consumption of either black tea (Clifford, Copeland, Bloxsidge, & Mitchell, 2000) or soft drinks (Fujii, Omori, Taguchi, & Ogata, 1991) by these subjects. Other compounds that may have been expected to be present in urine samples such as tyrosine, methylhippuric acids, oleuropein and hydroxytyrosol were not detected using total ion detection ESI (TIC; LC-MS) or PDA detection (HPLC). On the other hand, Phase II metabolites of the latter compounds were observed in some instances.

3.2.2 Profiles following acute ingestion

In the case of acute ingestion, total ion chromatograms from LC-MS analyses of the urine at baseline and one hour following supplement ingestion (data not shown) exhibited no distinct differences. Chromatograms (from HPLC analysis at 220 and 280 nm and Total ion chromatograms from LC-MS analysis) pertaining to samples
collected up to 24 h showed minor differences in profiles; but these differences were evidently not related to consumption of the supplements as none of the chromatographic peaks reflected the presence of oleuropein or oleuropein-related entities in the urine. The LC-MS detection limit for oleuropein was below 1.0 µg/mL and with a volume of collected urine of 400 mL over the 6 h following ingestion, then 400 µg biophenol is required for detection. This represents less than 1% of an ingested dose of 60 mg. Thus, if at least 1% of the ingested dose was absorbed and excreted in the urine as the un-metabolised material it would be detectable. It follows that no more than 1% of the ingested dose of oleuropein was absorbed and renally excreted unchanged in the urine.

Extracted ion monitoring by LC-MS showed the excretion of co-eluting glucuronide metabolites derived from oleuropein. The metabolites were seen in all urine samples collected up to 6 h after acute ingestion (99% of the total excreted was eliminated in the first 3 h; metabolites were not detected in samples collected at 12 and 24 h) but not in all urine samples collected after chronic administration of the supplements. The appearance of the metabolites was evidently related to the time of sample collection relative to supplement ingestion as this variable was not standardised.

3.2.3 Characterisation of metabolites by LC-QTOFMS

Further investigation of the glucuronide metabolites by higher resolution LC-QTOFMS showed that there were in fact five species; 2 isomeric forms of C_{25}H_{30}O_{14} (Compounds 1 and 2) and 3 isomeric forms of C_{25}H_{32}O_{14} (Compounds 3, 4 and 5) (Figure 2). The accuracy of the QTOFMS was excellent as evidenced by negative ion data errors of 1.09 ppm for the former (neutral mass 554.1636 compared to a
calculated mass of 554.1636; 99.98% mass match) and 0.36 ppm for the latter sets of isomers (neutral mass 556.1792 compared to a calculated mass of 556.1792; 99.99% mass match).

Collision induced dissociation of the metabolite molecular ions in the negative ion mode (m/z 553.1563 and 555.1716, respectively) provided structural information and the formation of metabolites 1 and 2 can be explained via the loss of glucose from oleuropein forming oleuropein aglycone followed by attachment of glucuronic acid. Figure 3 shows proposed structures. MS/MS spectra (Figure 4) show characteristic fragments at m/z 377.12 indicative of the loss of glucuronic acid from oleuropein aglycone, m/z 153.09 is indicative of a hydroxytyrosol moiety whilst m/z 241.07 is representative of elenolic acid. The spectra for the two isomers are highly similar but can be differentiated by the presence of the fragment at m/z 311.0748 in the spectra of 2. This fragment at m/z 311.0748 suggests that the glucuronide is attached to the hydroxytyrosol moiety of the oleuropein aglycone metabolite and that this fragment is formed by cleavage about the ester linkage yielding elenolic acid as shown in Figure 5. Similar cleavage in the negative ion mode has been reported about this ester linkage for the structurally similar demethyloleuropein (Savarese, De Marco, & Sacchi, 2007). The formation of the m/z 311 fragment is stabilised via resonance and is more likely formed when the glucuronic acid is attached to the meta-hydroxy group of hydroxytyrosol rather than at the para position.

Metabolites 3, 4 and 5 differ from that of 1 and 2 by an additional two hydrogen atoms. Spectra for 3, 4 and 5 are very similar (Figure 6); a fragment ion at m/z 379 indicates the loss of glucuronic acid, with the peak for glucuronic acid present at m/z
A peak at \( m/z \) 243 may arise from the ring-opened form of elenolic acid (the ring closed form gives a peak at \( m/z \) 241 in Figure 4). Loss of H\(_2\)O from this ion would give rise to the peak at \( m/z \) 228 and further loss of CO\(_2\) would lead to the peak at \( m/z \) 181. Only the presence of the fragment ion \( m/z \) 311.07 differentiates compounds 4 and 5 from isomer 3. As mentioned above, \( m/z \) 311.07 suggests the glucuronide is attached to the hydroxytyrosol moiety of the metabolite; thus, in the case of the first eluting isomer, the glucuronide is most likely attached to the elenolic acid derived moiety of the metabolite. Differentiation of the isomers with respect to variation in the elenolic acid derived moiety is more subtle, and may be explained by the presence of either an oxane ring, or opening of the oxane ring to yield an aldehydic oxygen. Similar structures have been reported by De Nino et al 2000 (De Nino et al., 2000). Possible structures for isomers 3, 4 and 5 are shown in Figure 7.

Attempts to isolate the 5 metabolites by preparative scale HPLC for definitive structural characterisation by nuclear magnetic resonance (NMR) were thwarted by the low concentrations and limited chromatographic resolution of the metabolites. Such complementary data is necessary for the definitive structural assignment of the 5 metabolites.

3.2.4 Metabolites arising from microflora degradation

No metabolite of colonic microflora origin was seen over the 24 h time period following oral or rectal administration of the supplement in this study.

4. Discussion

4.1 Characterisation of olive supplements
Olive leaves are known to be a rich source of biophenols both in terms of concentration and diversity of compounds (Erbay & Icier, 2010). In the two supplements investigated in this study, oleuropein was the dominant biophenol. Previous work by Ryan et al. (Ryan, Prenzler, Lavee, Antolovich, & Robards, 2003) showed that other biophenols were present in olive leaves at higher concentrations than oleuropein at certain maturity stages of the leaves. Furthermore, the content of oleuropein in olive leaves fluctuated substantially as the leaves grew and matured. Therefore it is not known whether the predominance of oleuropein in the supplements was due to careful selection of harvest dates or the method used to produce the extract. Although several biophenols have been reported in olive leaf, the strong antioxidant activity of leaf extracts is attributed to the presence of oleuropein (Briante et al., 2002), although synergistic interactions among biophenols are also likely to be important (Benavente-Garcia, Castillo, Lorente, Ortuno, & Del Rio, 2000).

4.2 Liquid chromatography profiles of urine samples

As there were no data on the possible metabolites of biophenols from the olive leaf supplements, we adopted a non-targeted approach to profiling urine. Such an approach led to complex HPLC chromatograms with 280 nm detection (Figure 1). While this non-targeted approach has disadvantages in terms of data analysis (i.e. non-targeted approaches generate large amounts of data, requiring time-consuming analysis), the main advantage is that a larger number of metabolites are detected in their native form, namely as they exist in urine. In contrast, enzymatic treatments modify the metabolites and much information is lost e.g. whether metabolites exist as glucuronides, sulphates, etc. In this study, detection at 280 nm was able to locate Phase II metabolites of olive biophenols in the urine samples of subjects who
chronically ingested the olive supplements. Based on this observation, efforts were made to characterise these metabolites following acute ingestion and using more sophisticated instrumentation (LC-QTOFMS).

LC-QTOFMS of urine samples taken after an acute dose of olive supplement revealed 5 metabolites of oleuropein aglycone. Each metabolite was found to be glucuronated, but at differing positions on the phenolic moiety. Both ring-closed forms (as in Figures 3 and 7) and ring-opened forms (as in Figure 7) of oleuropein aglycone have been reported (Obied et al., 2008). Given the structural similarity of these compounds to oleuropein, which was the major biophenol in the olive leaf extract supplements, it is assumed that they are derived from oleuropein. However, the possibility that they arose as metabolites of a minor undetected component in the extracts cannot be excluded. The use of labelled oleuropein in a supplement would be necessary to obtain conclusive proof of the origin of the metabolites.

The identification of these 5 metabolites is of significance for further research of olive products containing oleuropein. With few exceptions, the urinary excretion products of oleuropein have generally been assumed (Del Boccio et al., 2003) to be hydroxytyrosol and its glycosides, tyrosol, and elenolic acid, as well as unmodified oleuropein. With LC-MS techniques, these previously reported excretory products were not detected in the current study. A recent paper by Garcia-Villalba et al. (Garcia-Villalba et al., 2010) reports the tentative identification of more than 60 metabolites in a concentrated (20 fold) urine extract after olive oil consumption. A total of 32 Phase II metabolites were reported based on accurate mass data from ESI-TOFMS analysis. Searching our QTOFMS data for these 32 metabolites using the
reported accurate mass data was undertaken, and whilst extracted ion chromatograms (EICs) in both the positive and negative ion modes suggested the presence of some of these compounds, the corresponding mass spectra were not commensurate with expected fragmentation patterns. For example, two peaks at 1.86 and 2.18 min were evident in the EICs based on the accurate mass of hydroxytyrosol glucuronide \((C_{14}H_{18}O_9)\) in both positive ([M+H]\(^+\) and [M+Na]\(^+\)) and negative ion ([M-H]) modes. Spectra from both ionisation modes for both peaks (Figure 8) shows that there are no characteristic fragments indicative of the loss of glucuronide from hydroxytyrosol glucuronide (i.e. at \(m/z\) 153 in the negative ion and 155 in the positive ion). The peak at 1.86 min could be interpreted to be hydroxytyrosol disulfate \((C_8H_{10}O_{10}S_2)\) based on negative ion data ([M-H] \(\approx\) 329.0713; loss of 96.0721 to give \(m/z\) 232.9992) however this seems unlikely due to the associated error for the [M-H]\(^-\) ion (325 ppm). Nevertheless, the [M-H]\(^-\) molecular ion in the negative ion is confirmed by the [M+Na]\(^+\) adduct at \(m/z\) 353.1204 in the positive ion mode. Spectra for the peak at 2.18 min differ to that of the first eluting peak and is not thought to be hydroxytyrosol glucuronide nor hydroxytyrosol disulfate; the peak at 2.18 min cannot be tentatively identified.

4.3 Absorption and excretion of biophenol metabolites

The results presented above provide some insight into the absorption and excretion of olive biophenols derived from olive leaf supplements. Given the lack of oleuropein degradation products (and their Phase II metabolites) detected in urine, it is highly likely that oleuropein survives the stomach intact and is absorbed as the glycosylated compound in the small intestine. The acid stability of oleuropein was demonstrated \textit{in vitro} by Corona et al. (Corona et al., 2006) who did not see hydrolysis of oleuropein
in an acidic environment modelling the stomach. There are mixed reports in the literature as to whether oleuropein can be absorbed in the small intestine. Consistent with our results, Tripoli et al. (Tripoli et al., 2005) reported the absorption of oleuropein over the small intestine, possibly involving the use of a glucose transporter. Conversely, Corona et al. [9] found oleuropein not to be absorbed over a rat model of the small intestine (Corona et al., 2006), and the authors concluded that oleuropein must reach the colon to be subsequently degraded by the microflora (Corona et al., 2006). The difference in results may be due to different models of the small intestine. In any case, both reports involve rat models, which may not be applicable to human studies.

The possibility of colonic microflora involvement in the degradation and subsequent absorption of olive biophenols was also investigated. No metabolite of colonic microflora origin was seen over the 24 h time period following oral or rectal administration of the supplement in this study. While it could be argued that the supplement needed to be placed higher up into the colon to be degraded, this was deemed quite unlikely as rectal placement of a commonly used phenol, aspirin, when administered as a suppository, is degraded and absorbed in this same region. Metabolites of colonic microflora, when the supplement is consumed orally, would be expected to be excreted in the later urine collection times (12-24 h) and this was not seen. This longer time allowed for the metabolite excretion is due to the longer half-life and additional transit time through the intestine required for the phenols to reach the colonic microflora (Rechner et al., 2002; Scalbert & Williamson, 2000). It may be concluded that when administered either orally or rectally, the biophenols present in
olive leaf extract supplements, in particular oleuropein, are not degraded by the colonic microflora.

In a study where rats were fed a single does of oleuropein (100 mg/kg), Del Boccio et al. (Del Boccio et al., 2003) found that hydroxytyrosol and oleuropein were excreted in urine as glucuronide conjugates and also in their unmodified forms. Additionally, they found that oleuropein was present after 1 h in plasma also in unmodified form (i.e. as the glucoside). Therefore, it would appear that in rats, oleuropein is absorbed with the glucose intact, agreeing with Tripoli et al. (Tripoli et al., 2005). While similar excretion rates for oleuropein were found in the present study and in the study with rats, it must be emphasised that human studies should not be interpreted based on results from other organisms. In the case of olive biophenol metabolism, Visioli et al. (Visioli et al., 2003) demonstrated that hydroxytyrosol metabolism differed between humans and rats. Indeed with respect to excretionary metabolites, our in vivo results differ to those (Del Boccio et al., 2003) from in vitro studies using rats. Thus, the value of human clinical studies cannot be underestimated.

5. Conclusion

This study is the first to provide in vivo human data on the excretion of oleuropein from olive leaf supplements. Accordingly, there is a lack of published data suitable for direct comparison. The presence of the metabolites in urine indicates that oleuropein reaches systemic circulation (i.e., it is at least partially absorbed) and is metabolised in the human system. Thus, it satisfies the necessary condition for bioavailability of absorption. Further work is required to characterise oleuropein
metabolites in the blood and to study the effect of olive leaf supplement in a cohort with compromised health.

Acknowledgements
The provision of a scholarship (MK) by the EH Graham Centre, Charles Sturt University is gratefully acknowledged. Olive leaf supplements were donated by Olive Leaf Australia.
Reference List


acid after administration of sodium benzoate (Biological monitoring-1). Journal of the Food Hygienic Society of Japan, 32, 177-182.


Obied, H. K., Allen, M. S., Bedgood, D. R., Prenzler, P. D., Robards, K., &


Figure captions

Figure 1. Chromatograms of urine samples comparing detection at both 220 and 280 nm for a representative control at baseline (A and B, respectively) and following 28 days of supplementation (C and D at 220 and 280 nm, respectively). These chromatograms show the large variation in urinary profile, within a single individual.

Figure 2. Urine analysis by LC-QTOFMS (negative ion mode) after chronic ingestion of olive leaf supplement showing compounds 1-5. The total ion chromatogram is shown at the top whilst extracted ion chromatograms for m/z 553.1563 and m/z 555.1716 are shown in the middle and bottom chromatograms, respectively.

Figure 3. Proposed structures for compounds 1 (left) and 2 (right).

Figure 4. Negative-ion CID spectra for the ion m/z 553.1563 for compounds 1 (top) and 2 (bottom).

Figure 5. Proposed pathway for the formation of the m/z fragment at 311.07 in the negative ion spectrum of compound 2 (and by extension to compounds 4 and 5).

Figure 6. Negative-ion CID spectra for the ion m/z 555.1716 for compounds 3 (top), 4 (middle) and 5 (bottom).

Figure 7. Proposed structures for compounds 3 (top; two alternatives), 4 and 5 (bottom).
Figure 8. Spectra for peaks at 1.86 and 2.18 min which were present in EICs for hydroxytyrosol glucuronide in both negative (top two spectra; EIC at [M-H]⁻ 329.0878) and positive ion (bottom two spectra; EIC at [M+H]⁺ 331.1024 and [M+Na]⁺ 353.0843) modes.
Randomized controlled study of the urinary excretion of biophenols following acute and chronic intake of olive leaf supplements

Megan Kendall¹, Marijka Batterham², Damien L. Callahan³, Daniel Jardine⁴, Paul D. Prenzler¹, Kevin Robards¹ and Danielle Ryan¹

¹ Charles Sturt University, EH Graham Centre for Agricultural Innovation, School of Agricultural and Wine Sciences, Wagga Wagga 2678, Australia

² Centre for Statistical and Survey Methodology, University of Wollongong, Northfields Ave Wollongong 2522, Australia

³ Metabolomics Australia, School of Botany, The University of Melbourne, Victoria 3010, Australia

⁴ Flinders Analytical, Faculty of Science & Engineering, Flinders University, Adelaide SA 5001

Corresponding author
Dr Danielle Ryan
Charles Sturt University
School of Wine and Food Sciences
Locked Bag 588
Wagga Wagga 2678
AUSTRALIA
Phone +61-2-6933 4382
Fax +61-2-6933 2866
Email dryan@csu.edu.au
Abstract
Olive leaf supplement was characterised by HPLC and administered to healthy young adults over 28 d (3 tablets or equivalent liquid dose per day) or in a single bolus dose of 3 tablets (or liquid equivalent). Oleuropein was the major biophenol in the extracts. There are no data on the excretion of urinary end-products of the metabolism of the olive leaf biophenols. Following both chronic and acute ingestion neither oleuropein nor its hydrolysis product, hydroxytyrosol, were detected in urine samples. However, glucuronic acid conjugates derived from oleuropein aglycone were detected in all urine samples up to 6 h following acute ingestion. The data suggest that oleuropein is bioavailable, which is a necessary pre-condition for bioactivity.

Keywords: Adults; excretion; healthy; oleuropein; urine

1. Introduction
As the world becomes more diet conscious, there is an increasing interest in olive products. Olive oil has been consumed over many centuries and health benefits have been associated with its intake. It has been studied extensively in recent times and its bioactivity (separate from its favourable fatty acid profile) has generally been attributed to an antioxidant effect by the phenolic fraction including oleuropein derivatives and hydroxytyrosol (Tripoli et al., 2005). Bioavailability, which is generally a pre-requisite for bioactivity, is a function of the capacity to be absorbed by the body and reach systemic circulation (Scalbert, Manach, Morand, Remesy, & Jimenez, 2005). An exception to this requirement is a direct antioxidant (or other) effect in the gastrointestinal tract, as this is independent of any phenolic absorption.
The rapid absorption of phenolic compounds in a dose dependent manner following consumption of olive oil has been demonstrated (Visioli et al., 2000). Data on the metabolism and excretion of olive biophenols are limited to olive oil as the vehicle. Some of the earlier data are contradictory and confusing (Edgecombe, Stretch, & Hayball, 2000; Del Boccio et al., 2003; Ranalli et al., 2006; Tuck & Hayball, 2002; Vissers, Zock, Roodenburg, Leenen, & Katan, 2002; Corona et al., 2006). A possible interpretation is that oleuropein is degraded by colonic microflora to species including hydroxytyrosol with the latter being well absorbed in the intestine (Corona et al., 2006). Moreover, the unavailability of standards has meant that a number of studies incorporated hydrolysis steps in the analytical methodology and thus did not distinguish between free phenols and their conjugated metabolites. Other studies demonstrated that the phenols are extensively metabolised in the gut and liver and are thus excreted mainly as Phase II metabolites of hydroxytyrosol and tyrosol (Miro-Casas et al., 2003a; Miro-Casas et al., 2003b; Miro-Casas et al., 2001). Two recent studies (Garcia-Villalba et al., 2010; Khymenets et al., 2011) have confirmed these earlier findings and provided quantitative data. However, the source of these metabolites remains unclear in some instances. Answers to such questions are not facilitated because in some instances the phenolic profile of the administered oils was not characterised and in others it showed several phenols (Garcia-Villalba et al., 2010; Khymenets et al., 2011) but with limited or no oleuropein.

Olive leaf contains many of the same phenols as the oil but in much higher concentrations (Silva, Gomes, Leitao, Coelho, & Boas, 2006). However, limited data suggest that the absorption of phenols is dependent on the vehicle of administration (Visioli et al., 2003) and yet there has been very limited in vivo research conducted on
these materials. We recently reported (Kendall et al., 2009) on the oxidative status of a healthy young population following ingestion of olive leaf supplements. A range of biomarkers did not show any change in the oxidative status of the cohort following supplement consumption. It is unclear whether the lack of bioactivity in our earlier study (Kendall et al., 2009) was a result of the population studied, poor bioavailability of the olive leaf phenols or poor choice of biomarkers. Leaf extracts are distinguished not only by higher phenolic concentrations than in olive oil but also by the phenolic profile (vide infra). Before repeating the oxidative status trial on a compromised group such as those with metabolic syndrome or a chronic disease such as diabetes we wished to gather more information on the metabolism and excretion of the olive leaf biophenols. This paper reports on the excretion and metabolism of oleuropein following both chronic and acute ingestion of olive leaf extract supplements.

2. Materials and Methods

2.1 Reagents and olive leaf supplement

Reagents and standards were obtained as previously reported (Ryan et al., 2002). Olive leaf supplements were provided as a capsule (Blooms Health Products ‘Olive Leaf’ batch 336163 70284) and a liquid extract (‘Olive Leaf Extract – Natural Flavour’ batch 731X-6A; Olive Leaf Australia, Coominya, QLD, Australia).

2.2 Human subjects

This study was approved by the Ethics in Human Research Committee of Charles Sturt University. Subjects (23 female, 22 male) were recruited from a class of first year health science students from Charles Sturt University, Wagga Wagga (Australia). Participants were generally in good health and exclusion criteria included being pregnant or breast-feeding, having any major chronic disease, wearing a pacemaker.
smoking cigarettes regularly, or having food allergies/intolerances. The average age, weight and body mass index (BMI) of the participants was 20.2 ± 3.0 y, 70.5 ± 16.8 kg and 23.2 ± 4.4 kg/m², respectively. Participants were instructed to consume one supplement (one capsule, or one 5 mL measure of liquid) three times per day, for 28 d as previously described. Subjects (23 female, 22 male; average age = 19 years) were recruited from a class of first year health science students from Charles Sturt University, Wagga Wagga (Australia) and were instructed to consume one supplement (one capsule, or one 5 mL measure of liquid) three times per day, for 28 d as previously described (Kendall et al., 2009). A spot urine sample was supplied by each subject both at baseline and follow-up in sterile 70 mL containers. Collection time was not controlled. These samples were transferred to the laboratory within 30 min of voiding, and stored in a freezer at -20° C until analysis by HPLC and LC-MS.

For acute ingestion, four subjects (2 male, 2 female) orally ingested three olive leaf extract capsules, and baseline urine samples were collected, as well as spot samples up to 24 h post-ingestion. All urine excreted over the 24 h period was collected in sterilised collection jars and the volume measured. After a 24 h wash out period, the subjects consumed 15 mL of the liquid olive leaf extract and urine samples were collected as before. The procedure was replicated five times over two years.

To investigate the role of colonic microflora, rectal administration of the capsule extract was performed (in triplicate) on two healthy adult subjects (1 male, 1 female who had not presented with any illness in the previous 12 months OR who had not been treated with antibiotics in the preceding 12 months) by insertion as a suppository approximately 6-7 cm into the rectum. Correct positioning of the capsule was verified.
by a Registered Nurse who also confirmed that the capsule had melted after 15 min by
digital examination. The inserted sample was retained for a minimum of 4 h. Urine
samples were collected and analysed as before. To investigate the role of colonic
microflora, rectal administration of the capsule extract was performed (in triplicate)
on two subjects (1 male, 1 female) by insertion as a suppository approximately 12 cm
into the rectum. The inserted sample was retained for a minimum of 4 h. Urine
samples were collected and analysed as before.

2.3 High performance liquid chromatography
HPLC was performed on a Varian 9021 solvent delivery system equipped with a
Varian 9065 Polychrom UV diode array detector (190-367 nm). The system was
maintained in a controlled temperature room at 21 ± 1 °C. A flow rate of 0.6 mL/min
and an injection volume of 20 µL were used. Separation was performed by gradient
elution on a 100 mm x 4.6 mm i.d., 3 µm, Polaris C-18 column (Varian, Australia)
attached to a SecurityGuard guard cartridge (Phenomenex, Australia). Software used
for data processing was Star Polychrom version 5.2. The mobile phases were freshly
prepared and degassed under vacuum using Phenomenex Nylon 45 µm membranes
and sonicated in a Sanophon ultrasonic bath (Ultrasonic Industries Pty. Ltd., Sydney,
Australia) for 15 min prior to HPLC analysis.

Solvent A was a mixture of water: formic acid (100:1 v/v) and Solvent B was a
mixture of acetonitrile: water: formic acid (50:50:1 v/v/v). A multi-step linear
gradient analysis was used as follows: initial, 100% solvent A isocratic for 17 min;
reducing to 96% at 20 min then isocratic to 25 min; reducing to 85% at 30 min and to
75% at 40 min then isocratic to 45 min; reducing to 65% at 50 min, to 30% at 55 min
and then to 0% solvent A at 65 min. The system was equilibrated between runs for 20 min using the starting mobile phase composition.

Urine samples were analysed after filtration through a 0.45 μm filter (Advantec MFS, Inc., Dublin, CA, USA) with a sterile syringe. No other pre-treatment of samples was performed.

2.4 Liquid Chromatography-Mass Spectrometry

2.4.1 LC-MS

Preliminary analyses were performed on a Micromass Quattro micro tandem quadruple mass spectrometer (Waters, Manchester, UK) using electrospray ionisation (ESI). LC separation was provided by a Waters liquid chromatograph (Milford, USA), consisting of a 2695 Separation Module and 2487 dual wavelength UV detector operated at 240 and 280 nm. An SGE Wakosil C18 column (150 mm × 2 mm; 5μm) was used. Solvents and gradient program were as used for HPLC. An injection volume of 10 µL and a constant flow of 0.2 mL/min were used for each analysis. The entire flow from the LC was directed into the mass spectrometer. Data were acquired by the Masslynx data system for both the MS and UV data. The mass spectral data were acquired for four alternative scans; Scan 1: Positive ion mode, cone voltage 35 V; Scan 2: Positive ion mode, cone voltage 70 V; Scan 3: Negative ion mode, cone voltage 30 V; Scan 4: Negative ion mode, cone voltage 70 V. All scans were performed in the range 80 to 1500 m/z in 1 sec.

2.4.2 LC-QTOFMS
Samples were analysed by reversed phase liquid chromatography (LC; Agilent 1200) with QTOFMS (quadrupole time-of-flight mass spectrometry; Agilent 6520) detection in both positive and negative ion modes. Separation was achieved using an Agilent Zorbax XDB C18 column (100 mm x 2.1 mm i.d.; 1.8 µm) and the column temperature was 35 °C. Solvent A was formic acid in water (0.1%) and Solvent B was formic acid in acetonitrile (0.1 %). The gradient commenced at 2 % B increasing to 30 % B at 5.0 min and 100 % B at 10.0 min. The gradient was held at 100 % B for 2 mins before returning to 2 % B at 12.1 mins (held at 2 % B until 17 mins). A flow rate of 0.4 mL/min was used together with an injection volume of 5 µL.

Conditions for the ESI were: nebuliser pressure 40 psi; gas flow-rate 8 L/min; gas temperature 300 °C; capillary voltage 4000 V; fragmentor voltage 140 V and skimmer voltage 65 V. Collision induced dissociation (CID) in the negative ion mode was achieved using a collision energy of 20 V. A scan rate of 2 scans/s was used for both full scan and MS/MS analyses, and data was collected in the range of 100–1600 m/z. The mass accuracy of the QTOFMS is ± < 2 ppm.

3. Results

3.1 Characterisation of olive supplements

Characterisation of the capsule and liquid supplements using HPLC and LC-MS (Kendall, Batterham, Prenzler, Ryan, & Robards, 2008) showed a single major phenolic peak that was identified from retention and mass spectral data (retention time of 61.1 min, m/z 541 ESI⁺, m/z 539 ESI-) as oleuropein by comparison to a commercial standard. The oleuropein content of the capsule and liquid was calculated as 20 mg/tablet and 22 mg/5 mL, respectively, via external calibration using an
oleuropein standard. The liquid supplement was a much more heterogenous mixture when compared to the capsule supplement as evidenced by more peaks in the chromatograms (data not shown). A much smaller peak at 37.0 min in both capsule and liquid supplements was tentatively identified as hydroxytyrosol glucoside, based on previous work on olives (Ryan et al., 2002; Ryan, Robards, & Lavee, 1999) and olive waste (Obied et al., 2005).

3.2 Liquid chromatography profiles of urine samples
3.2.1 Profiles following chronic ingestion
HPLC profiling of the study participant’s urine samples at both baseline and following 28 d consumption was performed with photodiode array detection (PDA) monitoring at 220 and 280 nm. Selected chromatograms are shown in Figure 1. The chromatograms at 220 nm were dominated by a relatively small number of peaks with high intensity in contrast to the chromatograms generated at 280 nm which generally contained many peaks of comparable intensity. The major peaks in the 220 nm chromatograms included creatinine, uric acid and hippuric acid plus much lower concentrations of the 3- and 4-hydroxyhippuric acids. LC-MS plus use of commercial standards confirmed the presence of these compounds. Uric acid and creatinine were present in every sample and there was a semi-quantitative correlation with creatinine results from an assay kit (Kendall et al., 2009). Hippuric acid was detected in approximately 50% of urine samples with 1000-fold variation in concentration. This probably indicates consumption of either black tea (Clifford, Copeland, Bloxsidge, & Mitchell, 2000) or soft drinks (Fujii, Omori, Taguchi, & Ogata, 1991) by these subjects. Other compounds that may have been expected to be present in urine samples such as tyrosine, methylhippuric acids, oleuropein and hydroxytyrosol were
not detected using total ion detection ESI (TIC; LC-MS) or PDA detection (HPLC). On the other hand, Phase II metabolites of the latter compounds were observed in some instances.

3.2.2 Profiles following acute ingestion

In the case of acute ingestion, total ion chromatograms from LC-MS analyses of the urine at baseline and one hour following supplement ingestion (data not shown) exhibited no distinct differences. Minor differences in the samples collected up to 24 h were seen in chromatograms (from HPLC analysis) at 220 and 280 nm and in total ion chromatograms TIC (from LC-MS analysis); but these were evidently related to non-phenolic dietary components as none of the observable changes reflected the presence of oleuropein or oleuropein-related entities in the urine. Chromatograms (from HPLC analysis at 220 and 280 nm and Total ion chromatograms from LC-MS analysis) pertaining to samples collected up to 24 h showed minor differences in profiles; but these differences were evidently not related to consumption of the supplements as none of the chromatographic peaks reflected the presence of oleuropein or oleuropein-related entities in the urine. The LC-MS detection limit for oleuropein was below 1.0 µg/mL and with a volume of collected urine of 400 mL over the 6 h following ingestion, then 400 µg biophenol is required for detection. This represents less than 1% of an ingested dose of 60 mg. Thus, if at least 1% of the ingested dose was absorbed and excreted in the urine as the un-metabolised material it would be detectable. It follows that no more than 1% of the ingested dose of oleuropein was absorbed and renally excreted unchanged in the urine.
Extracted ion monitoring by LC-MS showed the excretion of co-eluting glucuronide metabolites derived from oleuropein. The metabolites were seen in all urine samples collected up to 6 h after acute ingestion (99% of the total excreted was eliminated in the first 3 h; metabolites were not detected in samples collected at 12 and 24 h) but not in all urine samples collected after chronic administration of the supplements. The appearance of the metabolites was evidently related to the time of sample collection relative to supplement ingestion as this variable was not standardised.

3.2.3 Characterisation of metabolites by LC-QTOFMS

Further investigation of the glucuronide metabolites by higher resolution LC-QTOFMS showed that there were in fact five species; 2 isomeric forms of C_{25}H_{30}O_{14} (Compounds 1 and 2) and 3 isomeric forms of C_{25}H_{32}O_{14} (Compounds 3, 4 and 5) (Figure 2). The accuracy of the QTOFMS was excellent as evidenced by negative ion data errors of 1.09 ppm for the former (neutral mass 554.1636 compared to a calculated mass of 554.1636; 99.98% mass match) and 0.36 ppm for the latter sets of isomers (neutral mass 556.1792 compared to a calculated mass of 556.1792; 99.99% mass match).

Collision induced dissociation of the metabolite molecular ions in the negative ion mode (m/z 553.1563 and 555.1716, respectively) provided structural information and the formation of metabolites 1 and 2 can be explained via the loss of glucose from oleuropein forming oleuropein aglycone followed by attachment of glucuronic acid. Figure 3 shows proposed structures. MS/MS spectra (Figure 4) show characteristic fragments at m/z 377.12 indicative of the loss of glucuronic acid from oleuropein aglycone, m/z 153.09 is indicative of a hydroxytyrosol moiety whilst m/z 241.07 is
representative of elenolic acid. The spectra for the two isomers are highly similar but can be differentiated by the presence of the fragment at m/z 311.0748 in the spectra of 2. This fragment at m/z 311.0748 suggests that the glucuronide is attached to the hydroxytyrosol moiety of the oleuropein aglycone metabolite and that this fragment is formed by cleavage about the ester linkage yielding elenolic acid as shown in Figure 5. Similar cleavage in the negative ion mode has been reported about this ester linkage for the structurally similar demethyloleuropein (Savarese, De Marco, & Sacchi, 2007). The formation of the m/z 311 fragment is stabilised via resonance and is more likely formed when the glucuronic acid is attached to the meta-hydroxy group of hydroxytyrosol rather than at the para position.

Metabolites 3, 4 and 5 differ from that of 1 and 2 by an additional two hydrogen atoms. Spectra for 3, 4 and 5 are very similar (Figure 6); a fragment ion at m/z 379 indicates the loss of glucuronic acid, with the peak for glucuronic acid present at m/z 175. A peak at m/z 243 may arise from the ring-opened form of elenolic acid (the ring closed form gives a peak at m/z 241 in Figure 4). Loss of H₂O from this ion would give rise to the peak at m/z 228 and further loss of CO₂ would lead to the peak at m/z 181. Only the presence of the fragment ion m/z 311.07 differentiates compounds 4 and 5 from isomer 3. As mentioned above, m/z 311.07 suggests the glucuronide is attached to the hydroxytyrosol moiety of the metabolite; thus, in the case of the first eluting isomer, the glucuronide is most likely attached to the elenolic acid derived moiety of the metabolite. Differentiation of the isomers with respect to variation in the elenolic acid derived moiety is more subtle, and may be explained by the presence of either an oxane ring, or opening of the oxane ring to yield an aldehydic oxygen.
Similar structures have been reported by De Nino et al 2000 (De Nino et al., 2000). Possible structures for isomers 3, 4 and 5 are shown in Figure 7.

Attempts to isolate the 5 metabolites by preparative scale HPLC for definitive structural characterisation by nuclear magnetic resonance (NMR) were thwarted by the low concentrations and limited chromatographic resolution of the metabolites. Such complementary data is necessary for the definitive structural assignment of the 5 metabolites.

3.2.4 Metabolites arising from microflora degradation

No metabolite of colonic microflora origin was seen over the 24 h time period following oral or rectal administration of the supplement in this study.

4. Discussion

4.1 Characterisation of olive supplements

Olive leaves are known to be a rich source of biophenols both in terms of concentration and diversity of compounds (Erbay & Icier, 2010). In the two supplements investigated in this study, oleuropein was the dominant biophenol. Previous work by Ryan et al. (Ryan, Prenzler, Lavee, Antolovich, & Robards, 2003) showed that other biophenols were present in olive leaves at higher concentrations than oleuropein at certain maturity stages of the leaves. Furthermore, the content of oleuropein in olive leaves fluctuated substantially as the leaves grew and matured. Therefore it is not known whether the predominance of oleuropein in the supplements was due to careful selection of harvest dates or the method used to produce the extract. Although several biophenols have been reported in olive leaf, the strong
antioxidant activity of leaf extracts is attributed to the presence of oleuropein (Briante et al., 2002), although synergistic interactions among biophenols are also likely to be important (Benavente-Garcia, Castillo, Lorente, Ortuno, & Del Rio, 2000).

4.2 Liquid chromatography profiles of urine samples

As there were no data on the possible metabolites of biophenols from the olive leaf supplements, we adopted a non-targeted approach to profiling urine. Such an approach led to complex HPLC chromatograms with 280 nm detection (Figure 1). While this non-targeted approach has disadvantages in terms of data analysis (i.e. non-targeted approaches generate large amounts of data, requiring time-consuming analysis), the main advantage is that a larger number of metabolites are detected in their native form, namely as they exist in urine. In contrast, enzymatic treatments modify the metabolites and much information is lost e.g. whether metabolites exist as glucuronides, sulphates, etc. In this study, detection at 280 nm was able to locate Phase II metabolites of olive biophenols in the urine samples of subjects who chronically ingested the olive supplements. Based on this observation, efforts were made to characterise these metabolites following acute ingestion and using more sophisticated instrumentation (LC-QTOFMS).

LC-QTOFMS of urine samples taken after an acute dose of olive supplement revealed 5 metabolites of oleuropein aglycone. Each metabolite was found to be glucuronated, but at differing positions on the phenolic moiety. Both ring-closed forms (as in Figures 3 and 7) and ring-opened forms (as in Figure 7) of oleuropein aglycone have been reported (Obied et al., 2008). Given the structural similarity of these compounds to oleuropein, which was the major biophenol in the olive leaf extract supplements, it...
is assumed that they are derived from oleuropein. However, the possibility that they arose as metabolites of a minor undetected component in the extracts cannot be excluded. The use of labelled oleuropein in a supplement would be necessary to obtain conclusive proof of the origin of the metabolites.

The identification of these 5 metabolites is of significance for further research of olive products containing oleuropein. With few exceptions, the urinary excretion products of oleuropein have generally been assumed (Del Boccio et al., 2003) to be hydroxytyrosol and its glycosides, tyrosol, and elenolic acid, as well as unmodified oleuropein. With LC-MS techniques, these previously reported excretory products were not detected in the current study. A recent paper by Garcia-Villalba et al. (Garcia-Villalba et al., 2010) reports the tentative identification of more than 60 metabolites in a concentrated (20 fold) urine extract after olive oil consumption. A total of 32 Phase II metabolites were reported based on accurate mass data from ESI-TOFMS analysis. Searching our QTOFMS data for these 32 metabolites using the reported accurate mass data was undertaken, and whilst extracted ion chromatograms (EICs) in both the positive and negative ion modes suggested the presence of some of these compounds, the corresponding mass spectra were not commensurate with expected fragmentation patterns. For example, two peaks at 1.86 and 2.18 min were evident in the EICs based on the accurate mass of hydroxytyrosol glucuronide (C₁₄H₁₈O₉) in both positive ([M+H]+ and [M+Na]+) and negative ion ([M-H]⁻) modes. Spectra from both ionisation modes for both peaks (Figure 8) shows that there are no characteristic fragments indicative of the loss of glucuronide from hydroxytyrosol glucuronide (i.e. at m/z 153 in the negative ion and 155 in the positive ion). The peak at 1.86 min could be interpreted to be hydroxytyrosol disulfate (C₈H₁₀O₁₀S₂) based on
negative ion data ([M-H]⁻ 329.0713; loss of 96.0721 to give m/z 232.9992) however this seems unlikely due to the associated error for the [M-H]⁻ ion (325 ppm). Nevertheless, the [M-H]⁻ molecular ion in the negative ion is confirmed by the [M+Na]⁺ adduct at m/z 353.1204 in the positive ion mode. Spectra for the peak at 2.18 min differ to that of the first eluting peak and is not thought to be hydroxytyrosol glucuronide nor hydroxytyrosol disulfate; the peak at 2.18 min cannot be tentatively identified.

4.3 Absorption and excretion of biophenol metabolites

The results presented above provide some insight into the absorption and excretion of olive biophenols derived from olive leaf supplements. Given the lack of oleuropein degradation products (and their Phase II metabolites) detected in urine, it is highly likely that oleuropein survives the stomach intact and is absorbed as the glycosylated compound in the small intestine. The acid stability of oleuropein was demonstrated in vitro by Corona et al. (Corona et al., 2006) who did not see hydrolysis of oleuropein in an acidic environment modelling the stomach. There are mixed reports in the literature as to whether oleuropein can be absorbed in the small intestine. Consistent with our results, Tripoli et al. (Tripoli et al., 2005) reported the absorption of oleuropein over the small intestine, possibly involving the use of a glucose transporter. Conversely, Corona et al. [9] found oleuropein not to be absorbed over a rat model of the small intestine (Corona et al., 2006), and the authors concluded that oleuropein must reach the colon to be subsequently degraded by the microflora (Corona et al., 2006). The difference in results may be due to different models of the small intestine. In any case, both reports involve rat models, which may not be applicable to human studies.
The possibility of colonic microflora involvement in the degradation and subsequent absorption of olive biophenols was also investigated. No metabolite of colonic microflora origin was seen over the 24 h time period following oral or rectal administration of the supplement in this study. While it could be argued that the supplement needed to be placed higher up into the colon to be degraded, this was deemed quite unlikely as rectal placement of a commonly used phenol, aspirin, when administered as a suppository, is degraded and absorbed in this same region. Metabolites of colonic microflora, when the supplement is consumed orally, would be expected to be excreted in the later urine collection times (12-24 h) and this was not seen. This longer time allowed for the metabolite excretion is due to the longer half-life and additional transit time through the intestine required for the phenols to reach the colonic microflora (Rechner et al., 2002; Scalbert & Williamson, 2000). It may be concluded that when administered either orally or rectally, the biophenols present in olive leaf extract supplements, in particular oleuropein, are not degraded by the colonic microflora.

In a study where rats were fed a single does of oleuropein (100 mg/kg), Del Boccio et al. (Del Boccio et al., 2003) found that hydroxytyrosol and oleuropein were excreted in urine as glucuronide conjugates and also in their unmodified forms. Additionally, they found that oleuropein was present after 1 h in plasma also in unmodified form (i.e. as the glucoside). Therefore, it would appear that in rats, oleuropein is absorbed with the glucose intact, agreeing with Tripoli et al. (Tripoli et al., 2005). While similar excretion rates for oleuropein were found in the present study and in the study with rats, it must be emphasised that human studies should not be interpreted based on
results from other organisms. In the case of olive biophenol metabolism, Visioli et al. (Visioli et al., 2003) demonstrated that hydroxytyrosol metabolism differed between humans and rats. Indeed with respect to excretionary metabolites, our in vivo results differ to those (Del Boccio et al., 2003) from in vitro studies using rats. Thus, the value of human clinical studies cannot be underestimated.

5. Conclusion

This study is the first to provide in vivo human data on the excretion of oleuropein from olive leaf supplements. Accordingly, there is a lack of published data suitable for direct comparison. The presence of the metabolites in urine indicates that oleuropein reaches systemic circulation (i.e., it is at least partially absorbed) and is metabolised in the human system. Thus, it satisfies the necessary condition for bioavailability of absorption. Further work is required to characterise oleuropein metabolites in the blood and to study the effect of olive leaf supplement in a cohort with compromised health.

Acknowledgements

The provision of a scholarship (MK) by the EH Graham Centre, Charles Sturt University is gratefully acknowledged. Olive leaf supplements were donated by Olive Leaf Australia.
Reference List


acid after administration of sodium benzoate (Biological monitoring-1).
Journal of the Food Hygienic Society of Japan, 32, 177-182.

Garcia-Villalba, R., Carrasco-Pancorbo, A., Nevedomskaya, E., Mayboroda, O. A.,
Exploratory analysis of human urine by LC-ESI-TOF MS after high intake of
olive oil: understanding the metabolism of polyphenols. Analytical and
Bioanalytical Chemistry, 398, 463-475.

Kendall, M., Batterham, M., Obied, H., Prenzler, P. D., Ryan, D., & Robards, K.
(2009). Zero effect of multiple dosage of olive leaf supplements on urinary

Nutritional methodologies and their use in inter-disciplinary antioxidant

Khymenets, O., Farre, M., Pujadas, M., Ortiz, E., Joglar, J., Covas, M. I., & de la
Torre, R. (2011). Direct analysis of glucuronidated metabolites of main olive
oil phenols in human urine after dietary consumption of virgin olive oil. Food

Miro-Casas, E., Albaladejo, M. F., Covas, M. I., Rodriguez, J. O., Colomer, E. M.,
Raventos, R. M. L., & De La Torre, R. (2001). Capillary gas chromatography-
mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in

Miro-Casas, E., Covas, M. I., Farre, M., Fito, M., Ortuno, J., Weinbrenner, T., Roset,
Chemistry, 49, 945-952.

Miro-Casas, E., Covas, M. I., Fito, M., Farre-Albadalejo, M., Marrugat, J., & De La
Torre, R. (2003b). Tyrosol and hydroxytyrosol are absorbed from moderate
and sustained doses of virgin olive oil in humans. European Journal of Clinical
Nutrition, 57, 186-190.

Obied, H. K., Allen, M. S., Bedgood, D. R., Prenzler, P. D., Robards, K., &


**Figure captions**

Figure 1. Chromatograms of urine samples comparing detection at both 220 and 280 nm for a representative control at baseline (A and B, respectively) and following 28 days of supplementation (C and D at 220 and 280 nm, respectively). These chromatograms show the large variation in urinary profile, within a single individual.

Figure 2. Urine analysis by LC-QTOFMS (negative ion mode) after chronic ingestion of olive leaf supplement showing compounds 1-5. The total ion chromatogram is shown at the top whilst extracted ion chromatograms for \( m/z \) 553.1563 and \( m/z \) 555.1716 are shown in the middle and bottom chromatograms, respectively.

Figure 3. Proposed structures for compounds 1 (left) and 2 (right).

Figure 4. Negative-ion CID spectra for the ion \( m/z \) 553.1563 for compounds 1 (top) and 2 (bottom).

Figure 5. Proposed pathway for the formation of the \( m/z \) fragment at 311.07 in the negative ion spectrum of compound 2 (and by extension to compounds 4 and 5).

Figure 6. Negative-ion CID spectra for the ion \( m/z \) 555.1716 for compounds 3 (top), 4 (middle) and 5 (bottom).

Figure 7. Proposed structures for compounds 3 (top; two alternatives), 4 and 5 (bottom).
Figure 8. Spectra for peaks at 1.86 and 2.18 min which were present in EICs for hydroxytyrosol glucuronide in both negative (top two spectra; EIC at [M-H]− 329.0878) and positive ion (bottom two spectra; EIC at [M+H]+ 331.1024 and [M+Na]+ 353.0843) modes.
Figure 1
Figure 2

Counts vs. Acquisition Time (min)
Figure 3
Figure 5

$m/z$ 311.07
Figure 8

Counts (%) vs. Mass-to-Charge (m/z)