Author: H. Obied, P. Prenzler and K. Robards
Title: Potent antioxidant biophenols from olive mill waste
Journal: Food Chemistry
ISSN: 0308-8146
Year: 2008
Volume: 111
Issue: 1
Pages: 171-178

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Elsevier Editorial System(tm) for Food Chemistry
Manuscript Draft

Manuscript Number: FOODCHEM-D-07-02376R1

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Article Type: Research Article

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Dear Sir,

Please accept the revised manuscript titled "Potent antioxidant biophenols from olive mill waste" by Obied *et al*. (manuscript number FOODCHEM-D-07-02376 1). We have attended to the reviewers' comments as detailed below. We trust that with these changes the manuscript will now be accepted for publication in Food Chemistry.

Yours sincerely,

Dr Hassan K. Obied
Corresponding author

Response to Reviewers

1- The sample used in the current study is a two-phase by-product (pomace) as described under the experimental section "line 93" and not olive mill waste water as the reviewer commented. However, we have added a description of the nature of the sample under the experimental section and one sentence to the introduction to clarify any possible misunderstanding.

Introduction: "This study focuses on two-phase mill waste as this is the main waste-stream from Australian mills."

Experimental: "Correggiola OMW (semisolid pomace)".

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Potent antioxidant biophenols from olive mill waste

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Keywords: Activity-guided fractionation; Verbascoside; Screening; Bioactivity.

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Abstract

Olive mill waste is rich in biophenols that can be extracted and applied as natural antioxidants for the food and pharmaceutical industries. Bioactivity-guided fractionation combines the use of bioassay and chromatographic separation for isolation of potent bioactive compounds from highly complex plant extracts such as OMW and avoids tedious purification and identification of inactive phytochemicals. Antioxidant activity-guided fractionation and sub-fractionation of Correggiola OMW extract using semi-preparative HPLC and multidimensional antioxidant screening, followed by isolation of the screening hits are described. Activity-guided fractionation using four different bioscreens revealed verbascoside and 3,4-DHPEA-DEDA as the most potent antioxidants in *Correggiola* OMW extracts.

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1. Introduction

The complexity of plant extracts is a major challenge for drug discovery from nature. When bioassays are used as detectors for identification of bioactive molecules from crude extracts, the potential to discover novel compounds is increased even from well-studied species (Waterman, 1998). Re-examination of plant extracts with new screening systems will continue to provide new bioactive molecules (Kupchan, 1971). The choice of a suitable bioassay to guide isolation and purification of bioactive molecules is crucial for the success of the activity-guided fractionation process (Kingston, 1996).

Few reports have utilized antioxidant activity-guided fractionation to study olive fruit (McDonald, Prenzler, Antolovich & Robards, 2001) and olive mill waste (OMW) extracts (Visioli, Romani, Mulinacci, Zarini, Conte, Vincieri & Galli, 1999) (Amro, Aburjai & Al-Khalil, 2002). While all of these studies applied multidimensional antioxidant bioassays, the applied fractionation techniques differed considerably. The work of Visioli et al. (Visioli, Romani, Mulinacci, Zarini, Conte, Vincieri & Galli, 1999) essentially targeted different OMW extracts, prepared with different chromatographic cleanup procedures, rather than applying a systematic fractionation scheme (Obied, Allen, Bedgood, Prenzler, Robards & Stockmann, 2005). Amro et al. (Amro, Aburjai & Al-Khalil, 2002) obtained various fractions by silica gel column chromatography, however, the absence of biophenol standards did not allow detailed composition analysis of different fractions. McDonald et al. (McDonald, Prenzler, Antolovich & Robards, 2001) used semi-preparative HPLC to prepare the required fractions and fraction composition was determined by HPLC-DAD and HPLC-MS,

nevertheless they made no attempt to sub-fractionate and isolate the bioactive constituents.

In this study, antioxidant activity-guided fractionation and sub-fractionation of Correggiola OMW extract (COE) was carried out by means of semi-preparative HPLC and multidimensional antioxidant screening. A method was developed for isolation and purification of the screening hits, bioactive compounds. This study focuses on two-phase mill waste as this is the main waste-stream from Australian mills.

2. Material and Methods

2.1. Chemical Reagents

Potassium phosphate monobasic; potassium hexacyanoferrate(III) (potassium ferricyanide); anhydrous ferric chloride ($FeCl_3$); 5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4',4''-disulfonic acid sodium salt (ferrozine); trichloroacetic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, linoleic acid, β-carotene and ferrous chloride tetrahydrate ($FeCl_2.4\ H_2O$) were purchased from Sigma-Aldrich (Sydney, Australia).

2.2. Samples

Correggiola OMW (semisolid pomace) samples from a commercial two-phase olive oil mill (Pieralisi, Italy) were obtained from “Riverina Olive Grove” (Wagga Wagga, NSW, Australia) on June 8, 2004.

2.3. Analytical scale HPLC-DAD

HPLC-DAD was performed with a Varian 9021 solvent delivery system equipped with a Varian 9065 Polychrom UV diode array detector (190-367 nm). Separation was performed by gradient elution on a Luna C-18(2) column, 5 µm particle size; (150 mm x 4.6 mm) (Phenomenex, Australia) attached to a SecurityGuard guard cartridge (Phenomenex, Australia). Analysis conditions were described previously (Obied, Allen, Bedgood, Prenzler & Robards, 2005).

2.4. HPLC-MS

A Micromass Quattro micro tandem quadrupole mass spectrometer (Waters, Manchester, UK) was used for the analysis of OMW samples. LC separation was provided by a Waters liquid chromatograph (Waters, 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. Aqueous formic acid (1%) and methanol + acetonitrile + formic acid (89.5+9.5+1 v/v) served as solvents A and B, respectively. Analysis conditions were described previously (Obied, Bedgood Jr., Prenzler & Robards, 2007a).

2.5. Preparation of the crude extract for fractionation

Extraction was performed with 10 g batches of freeze-dried OMW powder. Each 10 g batch was extracted with 30 mL extraction solvent i.e. aqueous methanol (80% v/v; pH 2, HCl) for 30 min at ambient temperature (20 ± 2 °C). After filtration, the raffinate was re-extracted with 20 mL of the same extraction solvent and filtered. The combined filtrate was defatted with *n*-hexane (40 mL × 2). The defatted extract was concentrated in a rotary evaporator for 1 h (at < 35 °C). The concentrated crude

extracts were combined and freeze-dried. The freeze-dried crude extract powder was reconstituted with 50% aqueous methanol to provide a concentration 1 g/mL. The reconstituted solution of the crude extract was referred to as COE, which was used for analyses, bioassays and fractionation.

2.6. Fractionation

The crude extract was filtered through a 0.45 μm PTFE syringe filter before separation. The system comprised a Perkin Elmer binary LC-pump 250, ProStar 325 UV-VIS detector (Varian, USA) with Prep dual path flow cell 9 mm × 1 mm, Varian 9300 autosampler (Varian, USA) with a 2000 μL sample loop, and a Gilson FC 203B fraction collector. Fractionation was performed on an Alltima C18 column (10 mm × 250 mm; 5 μm) connected to an Alltima Prep-Guard C18 (7 mm × 33 mm; 10 μm) guard column. Varian Star 6.2 software was used to control the autosampler and the UV-VIS detector (280 nm). Solvent A was water/methanol/acetic acid (90+10+1, v/v/v) and solvent B was methanol/acetonitrile/acetic acid (90+10+1, v/v/v). A stepwise linear gradient elution at constant flow rate (3.5 mL/min) was performed for a total run time of 45 min as follows: Starting from 85% solvent A and 15% solvent B increasing to 25% solvent B over 10 min, to 30% solvent B over 5 min, then isocratic for 5 min, increased to 40% solvent B over 10 min, to 100% solvent B over 5 min and maintained isocratic at 100% solvent B for 5 min. Equilibration time was at least 15 min between runs. The five collected fractions (A-E) (**Table 1**) were concentrated under vacuum to evaporate the organic solvents at 37 °C for 45 min. The aqueous residues were freeze-dried and stored at –20 °C in air-tight, amber glass containers until required.

146 *2.7. Sub-fractionation*

147 Fraction (D) was sub-fractionated using the same instrumental setup described above
148 but with a 1000 µL sample loop. Solvent A was water/acetonitrile/acetic acid
149 (95+5+1, v/v/v) and solvent B was methanol/acetonitrile/acetic acid (50+50+1, v/v/v).
150 A stepwise linear gradient elution at constant flow rate (4 mL/min) was performed for
151 a total run time 20 min as follows: starting from 75% solvent A and 25% solvent B
152 increasing to 30% solvent B over 2 min, then isocratic for 3 min, increased to 45%
153 solvent B over 5 min, to 60% solvent B over 5 min that increased to 100% solvent B
154 over 5 min and back to initial conditions over 5 min. Seven sub-fractions were
155 collected by monitoring the chromatograms at 280 nm allowing one major peak in
156 each sub-fraction. Sub-fractions (D1) to (D6) were concentrated under a stream of
157 nitrogen to remove the organic solvents before freeze-drying, while fraction (D7) was
158 dried to a constant weight under a stream of nitrogen without freeze-drying.
159

160 *2.8. Isolation of verbascoside and 3,4-DHPEA-DEDA*

161 *2.8.1. Solid-liquid extraction and liquid-liquid extraction*

162 Seven grams of freeze-dried powder were extracted with 40 mL extraction solvent,
163 aqueous methanol (80% v/v; pH 2, HCl), for 30 min at ambient temperature (20 ± 2
164 °C). After filtration, the raffinate was re-extracted with 30 mL of the same extraction
165 solvent for 15 min and filtered. The combined filtrate was defatted with *n*-hexane (40
166 mL × 2). The defatted extract was concentrated in a rotary evaporator for 30 min (at <
167 35 °C), reconstituted with 10 mL methanol/water/acetic acid (31+69+1, v/v/v) and
168 saturated with sodium chloride before being filtered through a GF/F filter. The filtrate
169 was extracted with ethyl acetate (15 mL × 4). The combined ethyl acetate extract was

dried over anhydrous sodium sulfate before solvent removal under vacuum at < 35 °C. The residue was reconstituted with methanol/water/acetic acid (31+69+1; v/v/v) and filtered through a 0.45 µm PTFE syringe filter. Reconstituted extracts were fractionated with SPE within less than 3 h. The ethyl acetate extract showed high instability with time even when stored at 4 °C (Obied, Karuso, Prenzler & Robards, 2007), so evaporation and reconstitution were performed without delay.

2.8.2. Fractionation by solid phase extraction

Strata-X^{TM} GIGA^{TM} (1 g, 12 mL) SPE tubes (Phenomenex, Australia) were conditioned with 20 mL methanol and then equilibrated with 20 mL water/acetic acid (100+1) v/v. Reconstituted extracts were loaded and early eluting biophenols were washed out with 30 mL methanol/water/acetic acid (31+69+1, v/v/v) and the tube was left to dry under vacuum for 1 min. The fraction containing verbascoside and 3,4-DHPEA-DEDA was eluted with 25 mL methanol/water/acetic acid (45+55+1, v/v/v). The SPE tube was then washed with methanol/acetonitrile/acetic acid (80+20+1, v/v/v). The flow rate throughout the whole SPE elution experiment was kept at 2 mL/min by adjusting the vacuum valve. The SPE eluents were monitored with analytical scale HPLC (Obied, Bedgood Jr., Prenzler & Robards, 2007a).

The fractions containing verbascoside and 3,4-DHPEA-DEDA were pooled and stored at 4 °C. Subsequently, removal of organic solvent was performed under vacuum at < 35 °C and the aqueous solution was freeze-dried.

2.8.3. Isolation and purification with semi-preparative scale HPLC

The freeze-dried fraction was reconstituted with aqueous methanol (80%), and filtered through a 0.2 μm syringe nylon filter (Phenomenex, Australia). Separation, isolation, and purification of the reconstituted and filtered SPE fractions was performed using the same semi-preparative HPLC setup described above using a 1000 μL injection loop. Solvent A was water/methanol (95+5, v/v) and solvent B was methanol/acetonitrile (50+50, v/v). A stepwise linear gradient elution at constant flow rate = 5 mL/min was performed for a total run time 25 min as follows: 75% solvent A and 25% solvent B isocratically for 3 min then to 30% solvent B over 3 min, then isocratic for 9 min, then to 35% solvent B over 5 min, and finally to 80% solvent B over 5 min.

The eluents in the collection tubes of the fraction collector were examined by analytical HPLC at 280 nm, before combining them. The organic solvents were removed under nitrogen current at ambient temperature and the remaining aqueous solution was freeze-dried.

Though the isolated compounds showed a single major peak (> 70%) in their chromatograms at 280 nm, a further purification with semi-preparative HPLC was carried out to attain higher purity (> 90%). The same instrumental semi-preparative HPLC setup described above was applied. Solvent A was water and solvent B was acetonitrile. A stepwise-linear gradient elution at constant flow rate (3 mL/min) was performed for a total run time 20 min as follows: 70% solvent A and 30% solvent B increasing to 40% solvent B over 5 min, then to 70% solvent B over 5 min, and return to 30% solvent B over 5 min.

215 *2.9. Determination of total phenols*

216 Folin-Ciocalteu reagent was used for determination of total phenols as described
217 previously (Obied, Bedgood Jr., Prenzler & Robards, 2007a).

218 *2.10. Antioxidant Bioassays*

219 Standards, freeze-dried COE, and freeze-dried fractions and sub-fractions were
220 dissolved in aqueous ethanol (50%) to obtain the required concentrations.

221 *2.10.1. DPPH radical scavenging activity*

222 Assay was performed as described earlier (Obied, Karuso, Prenzler & Robards, 2007).

223 *2.10.2. Beta-Carotene Bleaching Test (BCBT)*

224 Assay was performed as described earlier (Obied, Bedgood Jr., Prenzler & Robards,
225 2007a).

226 *2.10.3. Reduction power*

227 The ability to reduce Fe(III) to Fe(II) was used to measure the reducing power of
228 different samples according to the Oyaizu method (Oyaizu, 1988) as described by Yen
229 et al. (Yen, Duh & Tsai, 2002). Samples (500 μL) containing different concentrations
230 (1-28 ppm) in 50% ethanol were added to phosphate buffer (0.2 M, 2.5 mL) pH 6.6
231 and aqueous potassium ferricyanide $K_3[Fe(CN)_6]$ solution (1%, 2.5 mL). The reaction
232 mixture was incubated at 50 °C for 20 min. To stop the reaction, trichloroacetic acid
233 (10%, 2.5 mL) was added and the mixture was shaken and centrifuged at 3000 rpm
234 for 10 min. 5 mL was pipetted from the supernatant and added to 5 mL water in 15
235 mL screw-capped glass tubes then aqueous $FeCl_3$ (1%, 1 mL) was added and the tubes
236 well vortexed before reading the absorbance at 700 nm. An increase in absorbance

indicates increased reducing power (RP). The average values of three distinct measures of absorbance were plotted against final concentrations (ppm).

2.10.4. Metal chelation

The ferrous ion chelation properties of various fractions and phenolic standards were measured according to the method of Carter (Carter, 1971) as described by Dorman et al. (Dorman, Kosar, Kahlos, Holm & Hiltunen, 2003) without any modifications. Methanol (900 μL) was added to aqueous $FeCl_2.4\ H_2O$ (2 mM, 100 μL) and 200 μL of different concentrations (0.1, 1.0, 10.0 mg/mL) of the test sample in 50% ethanol were added. The mixture was shaken and incubated at room temperature for 5 min. Ferrozine (5 mM, 400 μL) was added and the reaction mixture was shaken and re-incubated at room temperature for 10 min. The absorbance was recorded at 562 nm, the lower the absorbance the higher the ferrous iron chelating activity. The chelation capacity was calculated according to the equation:

Chelation Capacity % = $[(A_c - A_s)/A_c] \times 100$

Where A_c = absorbance of iron-ferrozine complex and A_s = absorbance of test solution.

2.11. Statistical Analysis

All assays were performed in triplicate and averaged. Data analysis was performed by Microsoft Excel using Student's t test and results were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Correggiola Olive Mill Waste Extract (COE)

The dry weight of the freeze-dried powder represented 63 ± 2% of the fresh weight of OMW. Upon extraction of the freeze-dried OMW powder with the acidified hydroalcoholic extraction solvent, the extractable matter content was 21 ± 2% of the freeze-dried weight. Thus, one gram of the fresh OMW contained 132 mg extractable matter and total phenol content 15.8 mg GAE (gallic acid equivalents) by Folin-Ciocalteu reagent i.e. 25.1 mg GAE/g dry weight.

3. 2. Biophenol content and antioxidant activity of fractions

Fractions collected by semi-preparative HPLC were injected into analytical HPLC and chromatograms compared with COE to identify their biophenol content (Fig. 1). The collection scheme, appearance, weight, and total phenol content for different fractions are given in Table 1. Fraction (A) contained the non-retained and highly polar compounds eluting before hydroxytyrosol glucoside. It constituted one third of the COE on a weight basis and its phenol content was negligible as measured by Folin-Ciocalteu reagent. This was due to the limited cleanup strategy adopted in the current study. Though fraction (B) contained hydroxytyrosol, hydroxytyrosol glucoside, and tyrosol and other minor constituents (Obied, Bedgood Jr., Prenzler & Robards, 2007b), the total phenol content measured by Folin-Ciocalteu reagent was the second lowest (Table 1). This is most probably due to the high content of inactive matrix components. Fraction (D) which contained the two major biophenols in COE i.e. verbascoside and 3,4-DHPEA-DEDA had the highest total phenol content, followed by fraction (E) and fraction (C). The elution of red pigment throughout the

chromatogram supported our early findings about the polymeric nature of the color of OMW (Obied, Allen, Bedgood, Prenzler & Robards, 2005).

INSERT Fig. 1

INSERT Table 1

In light of the importance of multidimensional evaluation of antioxidant activity (Obied, Bedgood Jr., Prenzler & Robards, 2007a), the fractions were subjected to four antioxidant assays representing four different antioxidant mechanisms. The radical scavenging activity was measured by the ability of the fractions to scavenge the relatively stable nitrogen free radical DPPH (Table 2). In accord with the total phenol content, fraction (D) had the highest free radical scavenging activity followed by fraction (E) > Fraction (C) > COE >> fraction (B) > fraction (A). The difference in free radical scavenging activity between fraction (D) and fraction (E) was small, yet it was statistically significant ($p < 0.05$). The biophenolic standards examined in this study were more effective DPPH free radical scavengers compared to the fractions and the activity was in the order: caffeic acid > hydroxytyrosol > oleuropein.

INSERT Table 2

The chain terminator potential was measured by BCBT. Being an emulsion system, the activity in β-carotene/linoleic acid system is expected to be parallel to the lipophilicity of the fractions as partitioning into the oil micelles plays a paramount role (Obied, Bedgood Jr., Prenzler & Robards, 2007a). This assumption was valid

except for fraction (C); activity was in the order: fraction (E) > COE > fraction (D) >> fraction (B) > fraction (C) > fraction (A) (Table 2). The exceptionally low antioxidant activity of fraction (C) in BCBT can be attributed to the predominant glycosidic nature of its major components (Obied, Bedgood Jr., Prenzler & Robards, 2007b). The COE showed enhanced activity in BCBT, higher than the standards, which is similar to what has been reported before (Obied, Bedgood Jr., Prenzler & Robards, 2007a). The same order of activity for the standards was maintained as for DPPH radical scavenging activity, though at much higher concentrations.

The ability to reduce Fe(III) to Fe(II) was used as a measure for reductive capacities of samples and standards. Results for this test are represented graphically in Fig. 2 and Fig. 3. The activities of fractions and standards were parallel to DPPH radical scavenging activity.

INSERT Fig. 2

INSERT Fig. 3

The Carter method (Jadhav, Nimbalkar, Kulkarni & Madhavi, 1996), applied in the present study, measures the ability of olive biophenols to compete with ferrozine for chelating iron(II) ions. The outcome of the reaction between antioxidant, metal, and lipids is not easily predictable and will depend on the nature of all of these species as well as the system conditions such as pH (Carter, 1971). This test did not work for the standard biophenols or for the fractions under the reaction conditions applied in the current study. In both cases, the absorption of the Fe(II)/ferrozine in the presence of

334 the biophenols was higher than the absorption of the negative control
335 “Fe(II)/ferrozine”. The greenish blue colors formed upon addition of biophenol
336 standards to iron solution suggested a chelating effect. The situation for COE fractions
337 was further complicated by their red color. Two comments about the literature may be
338 made. Firstly, the ratio of “biophenol: iron(II)” is variable and no guidelines are
339 available; and secondly, the use of different solvents affect the pH and ionization
340 characteristics of biophenols and subsequently their chelating properties. To conclude,
341 the Carter method as described by Dorman et al. (Dorman, Kosar, Kahlos, Holm &
342 Hiltunen, 2003) was not suitable for measuring the chelation properties of biophenol
343 fractions (0.1-10 mg/mL) or biophenol standards hydroxytyrosol, caffeic acid, and
344 oleuropein (0.1 to 10 mM). The strongly interfering color of biophenol/Fe(II) complex
345 disfavor the use of this test for determining the chelating properties of biophenols.
346
347 It was concluded that fraction (D) was the most promising antioxidant fraction for
348 further study for potent antioxidant leads.
349

350 *3. 3. Phenol content and antioxidant activity of fraction (D) sub-fractions*

351 Fraction (D) was sub-fractionated on semi-preparative HPLC monitored at 280 nm.
352 The aim was to restrict each sub-fraction to one major peak which resulted in 7 sub-
353 fractions D1 to D7 (Fig. 4). The sub-fractions contained a maximum 5 mg. DPPH
354 radical scavenging and Folin-Ciocalteu assay were selected for bioscreening of the
355 sub-fractions (D1)-(D7). Sub-fractions (D2) and (D5) had the highest total phenol
356 content as measured by Folin-Ciocalteu assay and the most efficient DPPH radical
357 scavenging activity (Table 3). From retention time, UV spectra, and analytical scale
358 LC-MS the major peak in (D2) corresponded to verbascoside, and in (D5) to 3,4-

DHPEA-DEDA (Fig. 5). The blue and green colors (result from pH change) of sub-fractions were caused by the cyanic polymeric pigment that was found earlier (Obied, Allen, Bedgood, Prenzler & Robards, 2005).

INSERT Fig. 4

INSERT Table 3

INSERT Fig. 5

3.4. Isolation of screening hits

The bioactivity-guided fractionation resulted in identifying verbascoside and 3,4-DHPEA-DEDA as the most effective antioxidant biophenols in Correggiola OMW extract. A method was developed for isolation and purification of the two compounds (**Material and methods**). The method depends on liquid-liquid extraction using ethyl acetate, which efficiently recovered medium polarity small molecular weight biophenols from hydroalcoholic crude extracts and introduce a significant cleanup step to remove the olive pigment, highly hydrophilic and polymeric matrix components.

Semi-preparative HPLC was used for activity-guided fraction of COE with minimal cleanup procedures to avoid loss of any bioactive compounds. As noticed from Table 1 and Table 3, only 85% of the injected weight could be recovered, most probably due to retention of some matrix components on the HPLC column. Some compounds of the COE bound to the C18 stationary phase and could not be eluted even with 100% methanol or acetonitrile. Therefore in targeted isolation of biophenols, semi-preparative scale C18 RP-HPLC column was replaced by SPE disposable tubes for the fractionation of ethyl acetate extract. SPE with the polymeric sorbent Strata-X

allowed fractionation and cleanup of the ethyl acetate reconstituted extract before applying fractions to a C18 RP-HPLC column. C18-T, C18-E, C8 SPE cartridges were tried but Strata-X showed higher retention capacity and superior biophenol recovery. Successive application of HPLC for purification of isolated compounds produced chromatographically pure compounds as detected by HPLC-DAD.

Though analytical scale quantification showed high content of 3,4-DHPEA-DEDA, the recovery of verbascoside (2.6 g/kg OMW) on the semi-preparative scale was higher than 3,4-DHPEA-DEDA (2.3 g/Kg OMW) compounds due to its relative high stability.

The diastereomers of 3,4-DHPEA-DEDA were inseparable when the mobile phase contained acetic acid, while two peaks were observed when no acid was added to the mobile phase. This could be explained by the nucleophilic addition reaction of the methanol of the mobile phase to the carbonyl carbon adjacent to the chiral carbon in 3,4-DHPEA-DEDA forming (hemi)acetals that resulted in separation of the diastereomers. Acetals are not stable under acidic conditions; they dissociate forming the original aldehydes, thus in the presence of acetic acid one broad peak was always noticed (Fig. 6). The peak splitting phenomenon of 3,4-DHPEA-DEDA due to nucleophilic addition of methanol was reported before (Selvaggini, Servili, Urbani, Esposto, Taticchi & Montedoro, 2006).

INSERT Fig. 6

4. Conclusions

To conclude, verbascoside was identified as the most potent antioxidant followed by 3,4-DHPEA-DEDA in COE by means of activity-guided fractionation. A laboratory-

scale method for separation and purification of screening hits was developed. Verbascoside and 3,4-DHPEA-DEDA can be recovered from Australian OMW. 3,4-DHPEA-DEDA is not available commercially. The high yield of purified verbascoside from Australian OMW suggests potential economic use of OMW as a source of verbascoside. Currently, HPLC grade verbascoside is available commercially at 16,000 €/gram.

Acknowledgments

We wish to thank Mr Michael Loughlin for technical assistance. This research was funded by Rural Industries Research and Development Corporation and Charles Sturt University (Writing-up grant scheme).

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Figure Captions:

Fig. 1. Chromatograms of crude COE and fractions collected, using analytical scale HPLC with detection at 280 nm.

Fig. 2. Reducing power of biophenol standards and Correggiola OMW extract (COE): increase in absorbance indicates increasing reducing power.

Fig. 3. Reducing power of Correggiola OMW extract (COE) and its fractions (A-E): increase in absorbance indicates increasing reducing power.

Fig. 4. Chromatogram of fraction D at 280 nm on semi-preparative HPLC system demonstrating different sub-fractions.

Fig. 5. RP-HPLC chromatograms of fraction (D2) and fraction (D5) at 280 nm and identification of the major peak in each fraction by DAD and MS in negative ion mode NIM.

Fig. 6. Effect of presence of acetic acid in the mobile on the resolution of 3,4-DHPEA-DEDA diastereomers.

Figure 1

Fig. 1. Chromatograms of crude COE and fractions collected, using analytical scale HPLC.

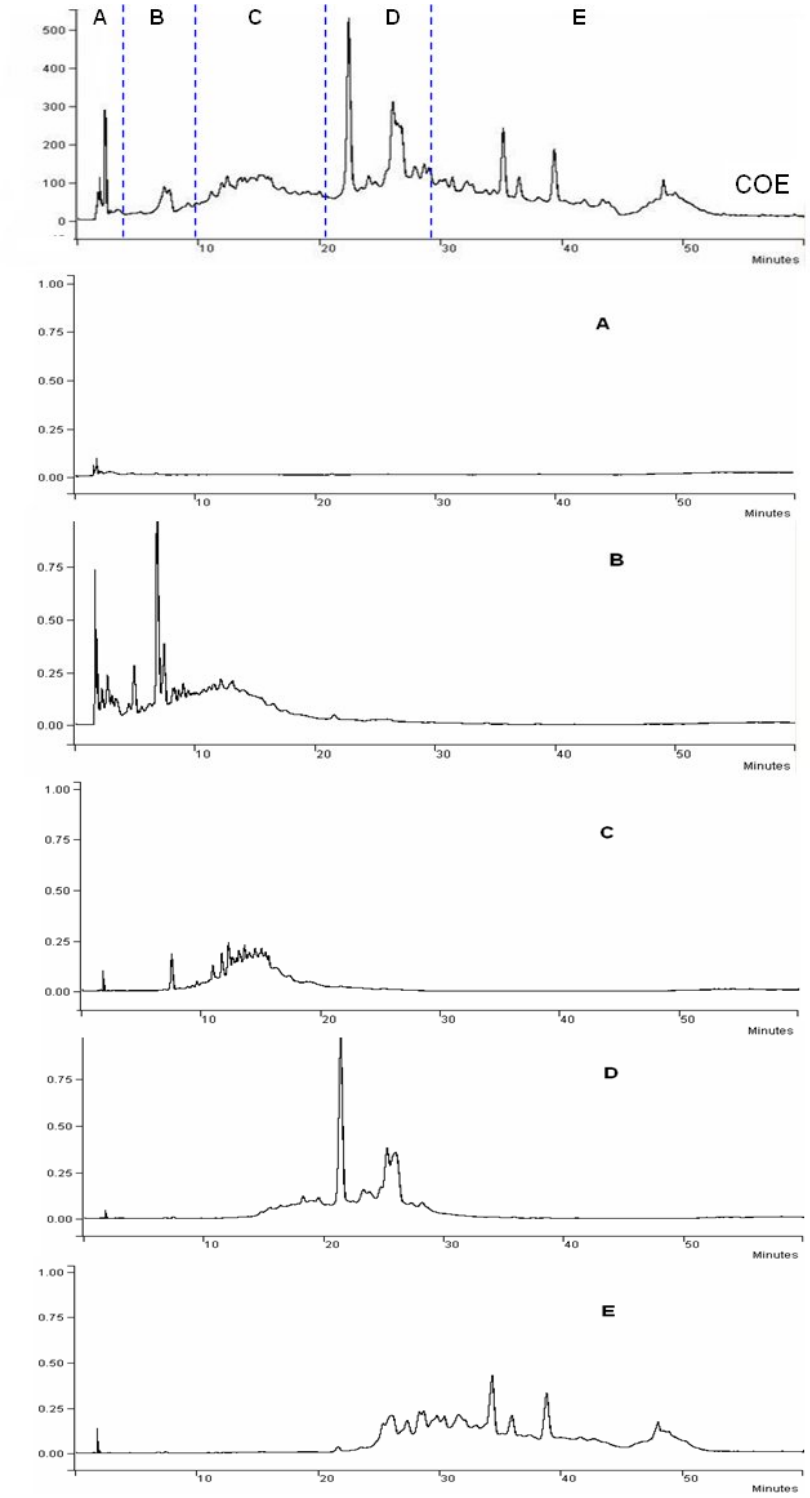


Figure 2

Fig. 2. Reducing power of biophenol standards and Correggiola OMW extract (COE): increase in absorbance indicates increasing reducing power.

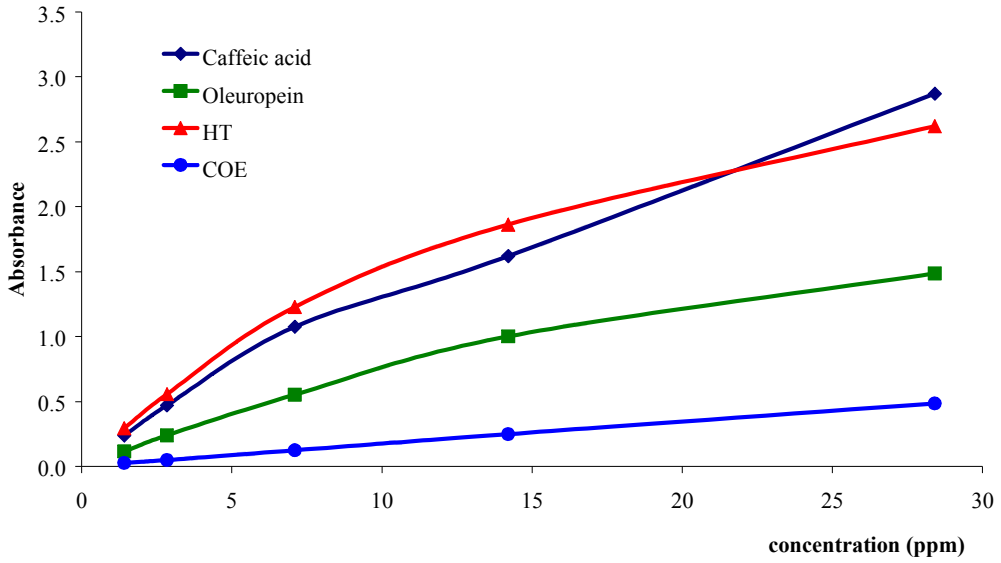


Figure 3

Fig. 3. Reducing power of Correggiola OMW extract (COE) and its fractions (A-E): increase in absorbance indicates increasing reducing power.

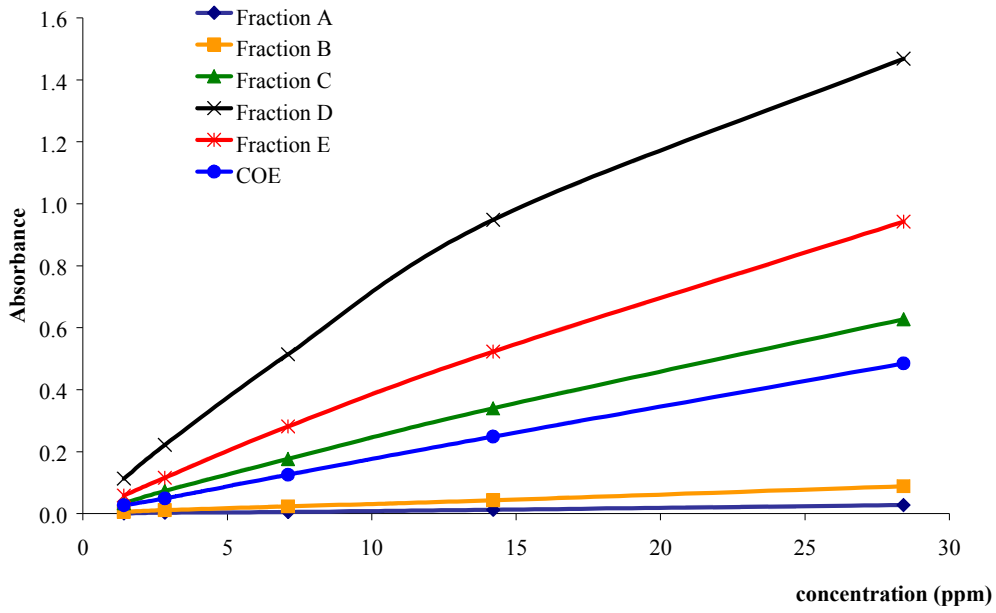


Figure 4

Fig. 4. Chromatogram of fraction D at 280 nm on semi-preparative HPLC system demonstrating different sub-fractions.

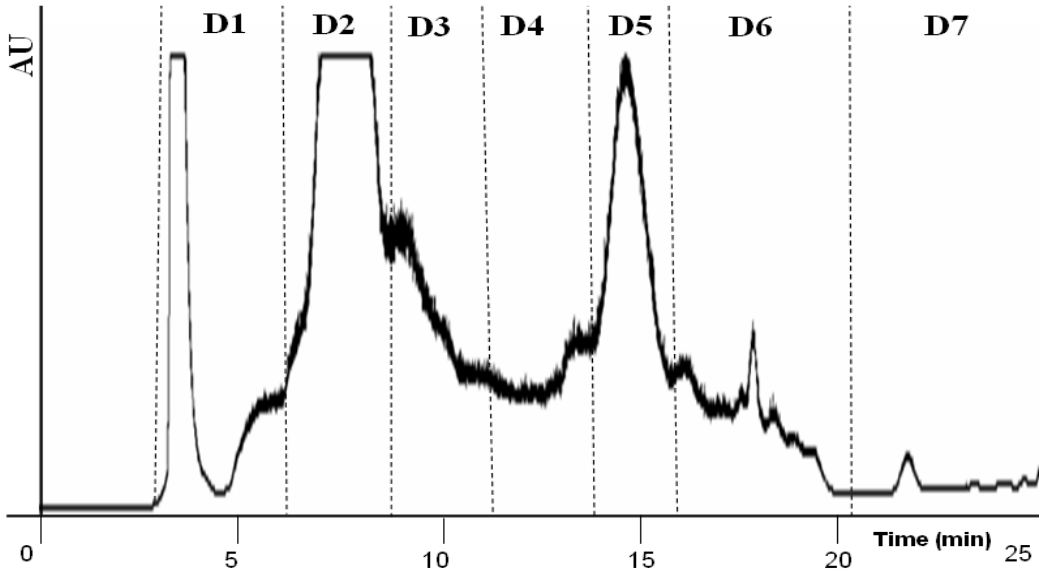


Figure 5

Fig. 5. RP-HPLC chromatograms of fraction (D2) and fraction (D5) at 280 nm and identification of the major peak in each fraction by DAD and MS in negative ion mode NIM.

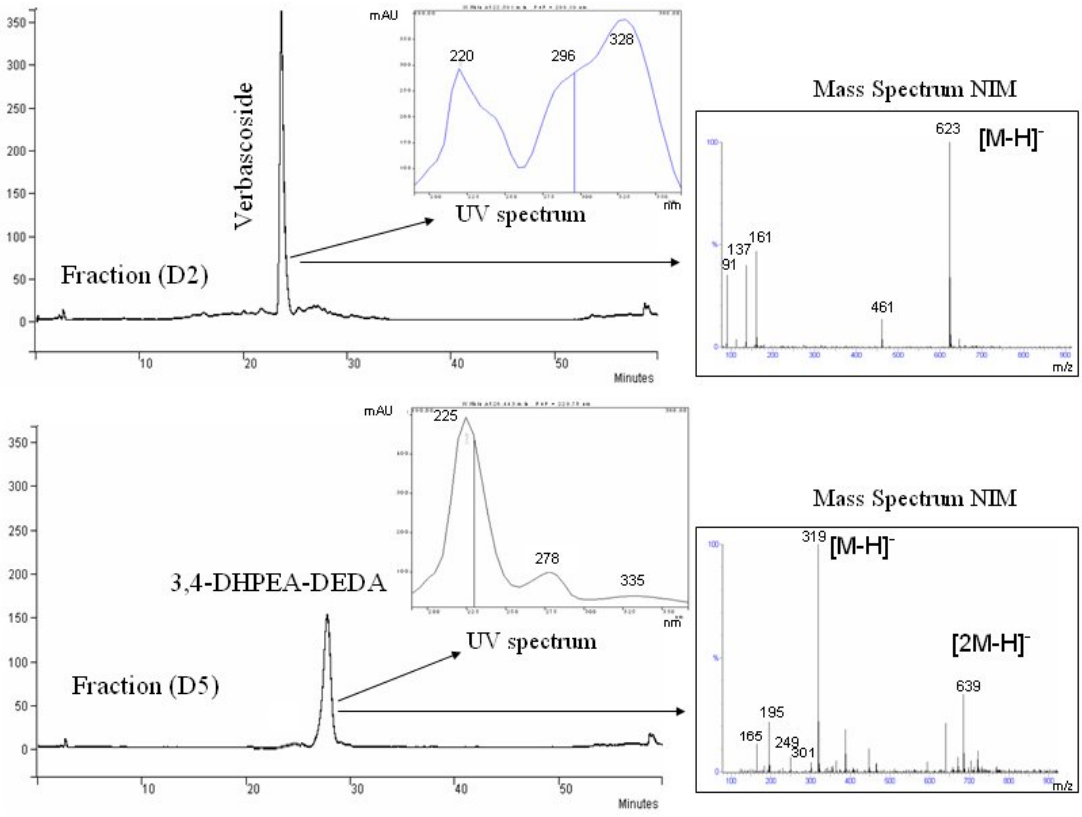
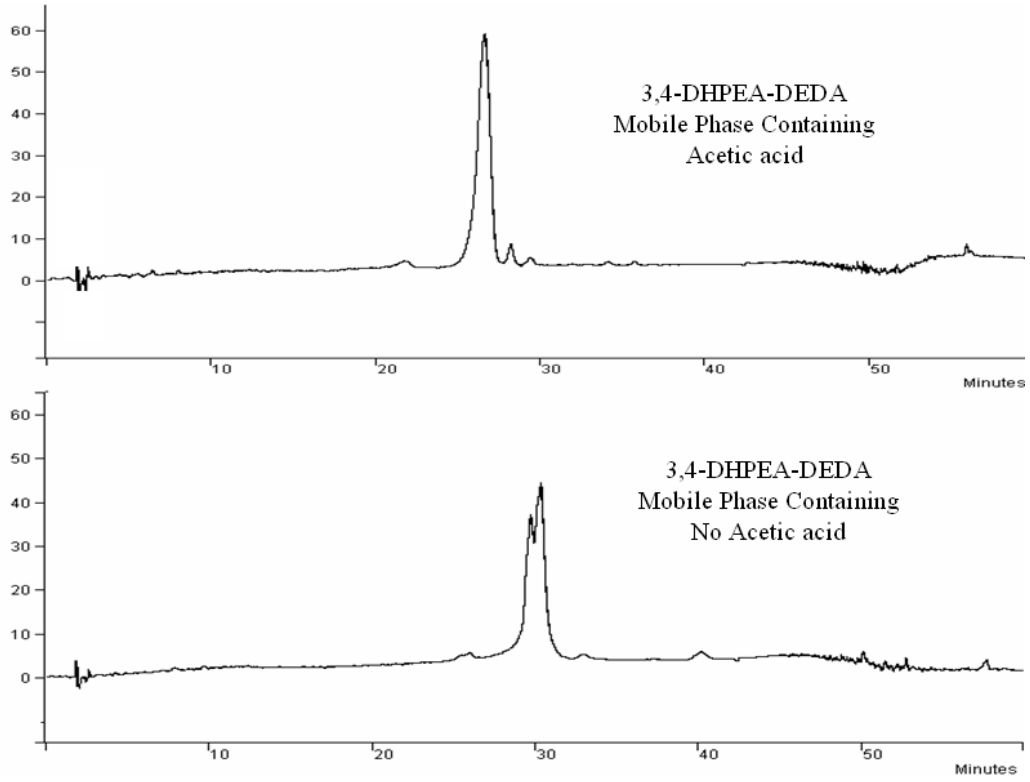


Figure 6

Fig. 6. Effect of presence of acetic acid in the mobile on the resolution of 3,4-DHPEA-DEDA diastereomers.



Table(s)

Table 1. Characterization of fractions collected by semi-preparative scale HPLC.

Fraction	**CT** (min)	**Color** [a]	**Appearance** [b]	**Wt %**	**TP** [c, d]
A	0- 3.7	colorless	pinkish dense powder	32	< LOQ
B	3.7-8.0	dark red	hygroscopic red powder	31	5.5
C	8.0-18	light red	red resinous	5	236.0
D	18-29	crimson red	hygroscopic red powder	7	457.7
E	29-45	dark red	dark red resinous	12	253.8
Summation of fractions				87	
COE				100	120.8

[a] color in solution upon collecting the fraction before freeze-drying; [b] freeze-dried product; [c] mg GAE/g; [d] coefficient of variation ≤ 10%

CT: collection time on semi-prep HPLC, Wt %: percentage of injected weight, LOQ: (Limit of Quantitation) = 0.007 mg GAE/g, COE: Correggiola OMW freeze-dried crude extract, TP: Total phenols measured by FC method

Table 2. Antioxidant activities of OMW crude extract and fractions and representative standards.

Sample	DPPH EC_{50} (ppm)	BCBT EC_{50} (ppm)
COE	38.5	48.0
Fraction A	642.0	456.1
Fraction B	214.7	163.7
Fraction C	25.2	443.4
Fraction D	10.0	60.8
Fraction E	11.1	26.0
Hydroxytyrosol	2.3	292.7
Caffeic acid	1.7	49.0
Oleuropein	7.6	341.0

Table(s)

Table 3. Characterization and antioxidant activity of Fraction (D) sub-fractions.

	Color in solution	Wt % [a]	TP [b]	DPPH EC_{50}
D1	Blue	14	176	12.8
D2	Bluish green	10	487	3.5
D3	Green	12	128	14.1
D4	Colorless	13	177	13.4
D5	Light blue	10	479	4.3
D6	Light blue	17	109	22.4
D7	Colorless	8	81	23.1

[a] % w/w of the injected weight into HPLC (excluding the void volume); total sum of the recovered biophenols = 84% of the injected amount.

[b] Total Phenol FC: mg GAE/g dry weight