JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Supplementation with Grape Seed Polyphenols Results in Increased Urinary Excretion of 3-Hydroxyphenylpropionic Acid, an Important Metabolite of Proanthocyanidins in Humans

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Grape seed extract provides a concentrated source of polyphenols, most of which are proanthocyanidins. Polymeric proanthocyanidins are poorly absorbed in the small intestine of humans, and exposure may result from metabolism to phenolic acids by colonic bacteria. Any biological effects of proanthocyanidins may be due to the phenolic acid metabolites. Several phenolic acids have been identified as proanthocyanidin metabolites, but these may be derived from a range of other dietary sources. The aim of this study was to determine if 24-h urinary excretion of specific phenolic acids increased significantly and consistently following regular supplementation with grape seed extract. In a randomized, double-blind placebo-controlled trial, 69 volunteers received grape seed extract (1000 mg/day total polyphenols) or placebo for 6 weeks. Supplementation with grape seed polyphenols resulted in a consistent increase in the excretion of 3-hydroxyphenylpropionic acid (3-HPP, $P < 0.001$) and 4-*O*-methylgallic acid ($P < 0.001$) and a less consistent increase in the excretion of 3-hydroxyphenylacetic acid ($P = 0.002$). The observed increase in 3-HPP is in line with the suggestion that this compound is a major phenolic acid breakdown product of proanthocyanidin metabolism in vivo.

**KEYWORDS: Grape seed polyphenols; proanthocyanidins; 3-hydroxyphenylpropionic acid**

## INTRODUCTION

Several lines of evidence support the suggestion that dietary polyphenols can contribute to reducing the risk of chronic diseases (*1*). Polyphenols are a group of phytochemicals with an estimated average intake of ∼1 g/day (*2*−*4*). The main classes of polyphenols are phenolic acids and flavonoids. Phenolic acids account for about one-third of the total intake and flavonoids about two-thirds (*2*). The contribution of various subclasses of flavonoids to total intake is uncertain and is likely to vary considerably between individuals. However, proanthocyanidins, which represent a diverse range of polymeric flavonoids (*2*), can provide a major contribution (50−500 mg/day) to total flavonoid intake (*5*−*7*).

Proanthocyanidins are present in a range of plant-derived foods and beverages (*2*, *6*−*9*). Grape seed extract can provide a concentrated source of polyphenols, most present as polymeric proanthocyanidins (*10*) with a wide range of galloylation (*7*, *11*). Available data on the absorption and metabolism of proanthocyanidins suggest negligible bioavailability of polymeric proanthocyanidins (*5*, *6*, *12*−*16*). Because of their high molecular weight, these compounds are poorly absorbed in the small intestine (*6*, *14*). Most proanthocyanidins will reach the colon, where colonic bacteria break them into smaller compounds including phenolic acids (*2*, *14*) (**Figure 1**). When these smaller compounds are absorbed, they may have biological effects.

In vitro studies of proanthocyanidins incubated with colonic microflora have identified several phenolic acids as proanthocyanidin metabolites, with 3-hydroxyphenylpropionic acid (3-HPP) a major metabolite (*5*). Many of these phenolic acids may also have metabolic precursors other than proanthocyanidins. The aim of the study was to determine if 24-h urinary excretion of specific phenolic acids increases following regular consumption of grape seed extract, containing predominately proanthocyandins.

## EXPERIMENTAL PROCEDURES

**Participants and Design.** Between February 2002 and May 2003, 74 hypertensive men and women were recruited from the Perth general population by the School of Medicine and Pharmacology at the University of Western Australia for a study designed to investigate the effect of supplementation with grape seed polyphenols and/or vitamin C on blood pressure. All volunteers were taking one or more antihypertensive drugs and remained on these for the duration of the study. All volunteers ceased any vitamin and antioxidant supplements, limited tea and coffee intake to <3 cups/day, and ceased drinking red wine and commercial fruit juice for at least 3 weeks prior to study

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10.1021/jf049404r CCC: $27.50 
Published on Web 07/24/2004

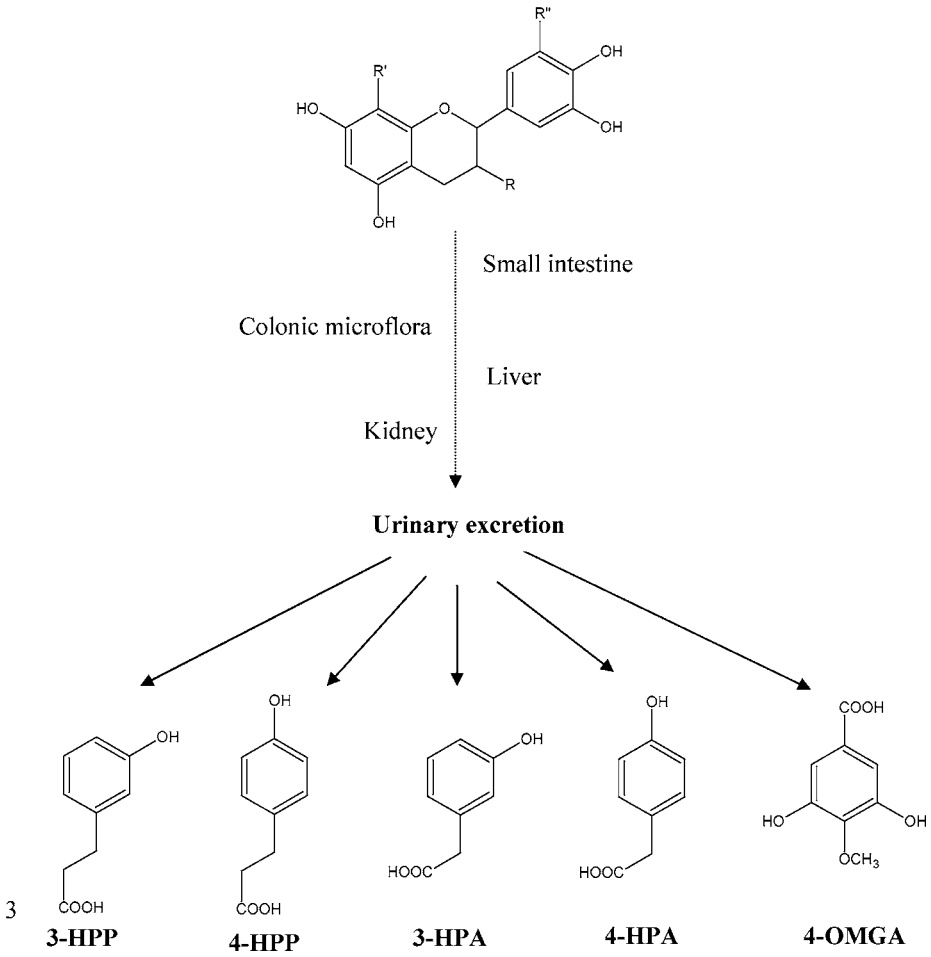


**Figure 1.** Urinary excretion of polyphenol metabolites. R = OH or gallate esters; R′ = flavonoid, H or OH; R″ = H or OH.

entry and for the duration of the study. Red wine and fruit juices may be important dietary sources of proanthocyanidins. Tea and coffee are important dietary sources of other polyphenols.

Participants were randomized to one of four supplementation groups: (i) 500 mg/day of vitamin C (2 × 250 mg, taken morning and evening) and 1000 mg/day of grape seed polyphenols (2 × 500 mg, taken morning and evening); (ii) 500 mg/day of vitamin C and matched grape seed polyphenol placebo; (iii) 1000 mg/day of grape seed polyphenols and matched vitamin C placebo; or (iv) matched placebo tablets for both grape seed polyphenols and vitamin C for 6 weeks in a double-blind fashion. All tablets were supplied by Taractechnologies (Nurioopta, South Australia). The total polyphenol concentration of the grape seed extract tablets was confirmed using a colorimetric assay that estimates total polyphenols in relation to a tannic acid standard curve (*16*). The tablet contained 20.5% polymeric compounds, with a mean degree of polymerization of 2.7, and the rest was made up of monomers, dimers, and trimers. Gallic acid was 0.05 wt %. The study was approved by the Royal Perth Hospital Human Ethics Committee. Written informed consent was obtained before inclusion in the study.

**Measurement of Urinary Phenolic Acids.** At baseline and 6 weeks postintervention, all participants provided a 24-h urine collection. Urine samples were stored at −80 °C until analysis. Concentrations of phenolic acids were measured in urine samples using a previously described method (*17*). Briefly, 1-hydroxy-2-naphthoic acid (50 ng, internal standard, purchased from Sigma Aldrich) was added to urine (750 μL) and acidified to pH 4.8 with dilute HCl. Thirty microliters of β-glucuronidase (3000 units of activity, Sigma catalog no. G7017) with sulfatase activity was added, mixed, and incubated at 37 °C for 12 h with occasional mixing. Most phenolic acids are present in urine as glucuronides with a small amount present as sulfates or in the free form. Samples were then extracted with ethyl acetate (2 mL), sodium bicarbonate (5% ww, 2 mL), and ethyl acetate again, dried under nitrogen, and derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, purchased from Sigma Aldrich) (50 μL) and pyridine (50 μL) at 40 °C for 30 min. The trimethylsilyl (TMS) derivatives were analyzed on a Hewlett-Packard HP 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer fitted with an HP5-MS cross-linked methyl silicone column (25m × 0.20 mm, 0.33 mm film thickness, Hewlett-Packard) using helium as the carrier gas. The major characteristic ions for the 3-HPP and 4-hydroxyphenylpropionic acid (4-HPP) diTMS (*m*/*z* 310, $M^+$), the 3-hydroxyphenylacetic acid (3-HPA) and 4-hydroxyphenylacetic acid (4-HPA) diTMS (*m*/*z* 296, $M^+$), the 4-*O*-methylgallic acid (4OMGA) diTMS (*m*/*z* 400, $M^+$), and the internal standard (*m*/*z* 317, $M^+ - 15$) were monitored. Peak identification was based on retention time and mass spectra compared with authentic standards [3-HPP was purchased from Lancaster; 3-HPA and 4-HPA were purchased from Sigma Aldric; and 4OMGA was synthesized (*17*)]. The 4-HPP was not routinely detectable, and data for this metabolite are not reported.

**Food Intake Assessment.** At baseline, all participants completed a validated food frequency questionnaire (*18*). Total fruit intake was coded from 1 to 6 according to the number of pieces of fruit eaten each day (1 = 0 pieces of fruit, 2 = <1, 3 = 1, 4 = 2, 5 = 3, 6 = ≥4). Total vegetable intake was coded from 1 to 7 according to the number of different vegetables eaten each day (1 = <1 vegetable, 2 = 1, 3 = 2, 4 = 3, 5 = 4, 6 = 5, 7 = ≥6). Information on usual vegetable serving size was also obtained using reference photographs and coded from 1 to 8. Individual foods were coded from 1 to 10 according to frequency of intake (1 = never, 2 = <1/month, 3 = 1−3/month, 4 = 1/week, 5 = 2/week, 6 = 3−4/week, 7 = 5−6/week, 8 = 1/day, 9 = 2/day, 10 = >3/day).

**Statistics.** Statistical analyses were performed using SPSS 11.5 software (Chicago, IL). There was no significant interaction between vitamin C and polyphenol treatments for effects on phenolic acid urinary

**Table 1.** Baseline and 6-Week 24-h Urinary Excretion of 3-Hydroxyphenylpropionic Acid (3-HPP), 3-Hydroxyphenylacetic Acid (3-HPA), 4-Hydroxyphenylacetic Acid (4-HPA), and 4-*O*-Methylgallic Acid (4OMGA), Phenolic Acid Breakdown Products of Proanthocyanidin Metabolism in Vivo, in 69 Participants following Supplementation with either Placebo or Grape Seed Polyphenols (1000 mg/Day)[a]

| | | mean (95% CI) | |
|---|---|---|---|
| phenolic acid | | placebo | polyphenols |
| 3-HPP | baseline | 803 (516, 1250) | 445 (306, 648) |
| ($\mu$g/24 h) | 6 weeks | 560 (343, 913) | 1071 (751, 1529)* |
| 3-HPA | baseline | 1392 (909, 2131) | 1189 (843, 1676) |
| ($\mu$g/24 h) | 6 weeks | 1026 (632, 1666) | 1722 (1109, 2674)† |
| 4-HPA | baseline | 976 (364, 2615) | 601 (228, 1583) |
| ($\mu$g/24 h) | 6 weeks | 1019 (371, 2800) | 313 (121, 807) |
| 4OMGA | baseline | 207 (122, 350) | 237 (137, 412) |
| ($\mu$g/24 h) | 6 weeks | 141 (83, 241) | 497 (362, 683)* |

[a]Significantly different versus placebo, following adjustment for baseline values: *, $P < 0.001$; †, $P = 0.002$

excretion, and therefore main effects analysis was used. Results are presented as differences between individuals on polyphenols versus those on placebo, regardless of whether they were also taking vitamin C. All phenolic acid data were non-normally distributed and were therefore log transformed prior to analysis. Results are presented as mean ± SEM, or geometric mean (95% confidence intervals), and $P < 0.05$ was the level of significance. Analysis of variance was used to assess potential differences between groups at baseline. Pearson's correlation coefficient ($r$) with two-tailed $P$ value was used to determine the degree and direction of association between variables. The effects of grape seed polyphenols on 6-week 24-h urinary excretion of phenolic acids were analyzed using general linear models after adjustment for baseline excretion of phenolic acids.

## RESULTS

Five participants did not complete the study; therefore, data on 69 participants (48 men and 21 women) who had a mean age of 61.6 ± 0.8 years and a mean body mass index (BMI) of 28.6 ± 0.4 kg/m$^2$ are presented. There were no differences between those assigned to placebo and those assigned to grape seed polyphenols for age, gender, or BMI. The excretion of phenolic acids was not different between men and women.

At baseline, 24-h urinary excretion of 3-HPP was associated with vegetable serving size ($r = 0.30$, $P = 0.01$), number of different vegetables eaten per day ($r = 0.30$, $P = 0.01$), and intake of legumes ($r = 0.39$, $P = 0.001$) and spinach ($r = 0.39$, $P = 0.001$). Excretion of 3-HPA was associated with vegetable serving size ($r = 0.25$, $P = 0.04$), number of different vegetables eaten per day ($r = 0.32$, $P = 0.02$), number of pieces of fruit eaten per day ($r = 0.25$, $P = 0.04$), and intake of legumes ($r = 0.32$, $P = 0.008$) and apples ($r = 0.41$, $P < 0.001$). Excretion of 4OMGA was positively associated with the number of cups of tea per day ($r = 0.60$, $P < 0.001$). Excretion of 4OMGA and 4-HPA was not associated with vegetable, legume, or fruit intake.

Supplementation with grape seed polyphenols resulted in a significant increase in 24-h urinary excretion of 3-HPP, 3-HPA, and 4OMGA, but not 4-HPA (**Table 1**). Within the group receiving the grape seed polyphenols, the 24-h urinary excretion of 3-HPP increased in all but four participants (**Figure 2A**). There was wide variation in the level of increase in 3-HPP between participants for the fixed dose (1000 mg/day) of grape seed polyphenols. The increase in 4OMGA was highly significant, with increases found in all but 6 of 32 participants (**Figure 2B**). Similarly, there was a significant increase in 3-HPA, but

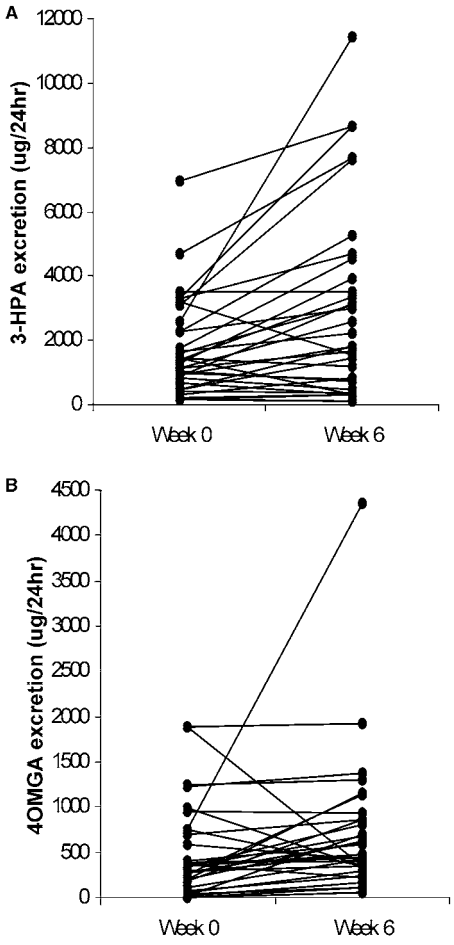


**Figure 2.** 24-h urinary excretion of 3-hydroxyphenylpropionic acid (**A**) and 4-*O*-methylgallic acid (**B**) in the 32 participants assigned to grape seed polyphenols (1000 mg/day) at baseline (week 0) and following 6 weeks of supplementation (week 6).

the observed changes within the group taking the polyphenols were not as consistent: increases were found in 22 of 32 participants and decreases in 10 of 32 (results not presented). Changes in 4-HPA were not significant or consistent (results not presented).

## DISCUSSION

3-HPP has previously been identified as the major in vitro metabolite of proanthocyanidins incubated with human colonic microflora (*5*). Increases in urinary excretion of 3-HPP have also been found after ingestion of proanthocyanidins in rats (*14*) and humans (*16*). We have now shown that 3-HPP increases significantly and consistently following supplementation with a grape seed extract rich in proanthocyanidins. Excretion of 3-HPP increased in all but four participants taking the grape seed polyphenols. In addition, a significant, but less consistent, increase in 3-HPA was found. Excretion of 4-HPA was not altered following supplementation with grape seed polyphenols.

There has been considerable research interest in the potential for dietary polyphenols to reduce the risk of chronic diseases. Polyphenols, which are the most abundant antioxidants in the human diet (*2*), are suggested to protect against oxidative stress in vivo and thereby reduce the risk of a range of chronic diseases. For example, results of in vitro studies and studies in animal models (*19*), cross-sectional and prospective population studies (*20*−*23*), and clinical intervention trials (*24*−*26*) are

consistent with the proposed benefits of dietary flavonoids on cardiovascular disease risk. Largely indirect evidence also indicates that polyphenols may reduce the risk of other chronic diseases including cancers (*27*, *28*). In addition, there is some evidence that foods, such as chocolate (*29*, *30*), and supplements, such as grape seed extract (*31*, *32*) and Pycnogenol (*33*, *34*), rich in proanthocyanidins, may reduce the risk of cardiovascular disease via a range of mechanisms.

Proanthocyanidins can provide a major contribution to total polyphenol intake (*5*, *6*). Given that polymeric proanthocyanidins are poorly absorbed (*6*, *13*, *14*), it may be that the absorbed colonic bacterial metabolites have the potential to exert biological effects. A major pathway for the metabolism of proanthocyanidins involves the breakdown of proanthocyanidins by colonic bacteria into smaller compounds including phenolic acids (*2*, *14*). The smaller compounds may then be absorbed and have biological effects. Observed in vitro activities of polymeric proanthyocyanidins may be unrelated to in vivo effects. Apart from exposure in the gastrointestinal tract, humans may not be exposed to these compounds in the circulation. Any biological effects of ingestion of polymeric proanthocyanidins may relate more to exposure to metabolites than to the compounds themselves.

Our results are in keeping with the potential for use of 3-HPP as a marker of proanthocyanidin intake. However, 3-HPP can be derived from other flavonoids (*35*), which may limit its use as a marker for proanthocyanidins. The less consistent increase in 3-HPA and the lack of a significant increase in 4-HPA are in keeping with the results of previous studies, which would suggest that these compounds are minor metabolites of proanthocyanidin breakdown (*5*, *14*). Our results also show that 3-HPP may be useful for the assessment of compliance in intervention trials using proanthocyanidins. An important aspect of such trials is to confirm that polyphenols in foods, beverages, or supplements are being consumed and absorbed, that is, to determine that volunteers have complied and been exposed to the polyphenols of interest.

We also observed a significant, and consistent, increase in 4OMGA following regular ingestion of grape seed polyphenols. These results suggest that the grape seed polyphenols are metabolic precursors of 4OMGA. Gallic acid may be released from larger flavonoids via esterase activity of colonic microflora (*36*). Given that many grape seed proanthocyanidins are galloylated (*11*), this may be the primary source of 4OMGA derived from the grape seed polyphenol supplement. However, 4OMGA may not be useful as a biomarker of proanthocyanidin exposure. The major metabolic precursor of 4OMGA is known to be free gallic acid (*17*). Other dietary components rich in free gallic acid, such as black tea, result in a greater increase in 4OMGA than the grape seed polyphenols. We observed an ~3-fold increase in 4OMGA following 1000 mg/day grape seed polyphenols, with a mean excretion of 483 (317, 735) $\mu$g/24 h. Five cups per day of black tea (~1000 mg/day of total polyphenols) results in a 24-h excretion of ~1000−2000 $\mu$g (*17*). Despite limiting tea intake to <3 cups/day in the present study, we found a significant and strong positive association between tea intake and 4OMGA at baseline.

In conclusion, the observed significant and consistent increases in 3-HPP and 4OMGA following supplementation with grape seed extract rich in proanthocyanidins are consistent with the suggestion that these phenolic acids are major metabolites of proanthocyanidin metabolism in humans. Measurement of 3-HPP and of 4OMGA may prove to be useful to investigate effects of dietary proanthocyanidins, and possibly other flavonoids, on chronic disease.

## ABBREVIATIONS USED

3-HPP, 3-hydroxyphenylpropionic acid; 4-HPP, 4-hydroxyphenylpropionic acid; 3-HPA, 3-hydroxyphenylacetic acid; 4-HPA, 4-hydroxyphenylacetic acid; 4OMGA, 4-*O*-methylgallic acid.

## ACKNOWLEDGMENT

We thank Kitiya Dufall for performing measurements of phenolic acid concentrations in urine samples and the volunteers who took part. Grape seed polyphenol and placebo tablets were provided by taractechnologies.

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**Received for review April 14, 2004. Revised manuscript received June 18, 2004. Accepted June 18, 2004. This project was supported by a National Health and Medical Research Council grant and a University of Western Australia Small Grant. N.C.W. gratefully acknowledges the assistance of a University of Western Australia Postgraduate Award.**

JF049404R