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**Dietary flavonols contribute to false-positive elevation of homovanillic acid, a marker of catecholamine-secreting tumours.**

**Authors:**

Emilie Combet[1], Mike EJ Lean[1], James G Boyle[2], Alan Crozier[1], D Fraser Davidson[3]

**Affiliations:**

[1] Life-course Nutrition and Health, Centre for Population and Health Sciences, University of Glasgow, Glasgow, G3 8SJ UK.

[2] School of Medicine, Royal Infirmary, Glasgow, G4 0SF UK.

[3] Biochemistry Department, Crosshouse Hospital, Kilmarnock, KA2 0BE UK.

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**Corresponding author:**

Prof MEJ Lean, Centre for Population and Health Sciences, Walton Building Level 4, Royal Infirmary, Glasgow G4 0SF, Scotland UK

Tel: +44 141 211 4686; Fax: +44 141 211 4844

Email: m.lean@clinmed.gla.ac.uk

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**Abstract**

Background: Urinary homovanillic acid (HVA) measurement is used routinely as a marker of first test for the screening of catecholamine-secreting tumours and dopamine metabolism, but generates a large number of false-positive results. With no guidelines for dietary restrictions prior to the test, we hypothesize that consumption of flavonol-rich foods (such as onions, tomatoes, tea) prior to urinary catecholamine screening could be responsible for false-positive urinary HVA in healthy subjects. Methods: A randomized, crossover dietary intervention was carried out in healthy subjects (n=17). Volunteers followed either a low or high-flavonol diet, for a duration of three days, prior to providing a 24-hour urine sample for HVA measurement using a routine, validated liquid chromatography method as well as a gas chromatography-mass spectrometry method. Results: Dietary flavonol intake significantly increased urinary HVA excretion ($p<0.001$), with 3 out of 17 volunteers (20%) exceeding the 40 µmoles/24 h upper limit of normal for HVA excretion (false-positive result). Conclusion: Dietary flavonols commonly found in foodstuff such as tomatoes, onions, and tea, interfered with the routine urinary HVA screening test and should be avoided in the three-day run-up to the test.

## INTRODUCTION:

Catecholamine-secreting tumours, including phaeochromocytoma, neuroblastoma and carcinoid tumours, are conventionally diagnosed by urinary screening for excess excretion of catecholamines and their metabolites [1]. Dopamine which is secreted by these tumours, including some form of malignant phaeochromocytoma [2, 3], is degraded via the monoamine oxidase (MAO) pathway, leading to the formation of phenolic acid metabolite 3,4-dihydroxyphenylacetic acid, which is further metabolized by catechol-O-methyltransferase to 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) [1]. The routine measurement of HVA involves chromatographic methods such as high performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS) [4-8], and the rarely used antibody-based techniques [9]. Certain drugs, e.g. L-dopa, are known to confound HVA estimation [10], as well as several dietary factors including vitamin B6 in combination with magnesium [11], nicotinamide adenine dinucleotide (NADH) used to treat patients with Parkinson's Disease [12], high monoamine meals in patients with Schizophrenia [13], and tyrosine supplementation in obese women [14]. These possible confounders are avoided prior to testing, under routine protocol.

A proportion of urinary HVA tests, commonly used as a first-stage screening test in the diagnosis of catecholamine-producing tumours, is high even when the patient is ultimately found not to have either a tumour or a recognized drug interference – i.e. an unexplained false-positive result [15]. This problem affects, in Scotland alone, as many as 30 adult and child patients per annum, who may then submitted to a series of further biochemical tests, as well as CT, MRI which ultimately reveal no tumour but lead to unnecessary expenditure by health services and occasioning high-levels of stress in patients referred for procedures which are not ultimately required [16].

We hypothesized that dietary factors may interfere with the interpretation of the HVA assay findings: HVA is also formed as a metabolite of dietary quercetin-based flavonoids when they reach the large intestine [17]. Rutin (quercetin-3-O-rutinoside), found in tea, tomatoes, apples, asparagus,

peaches, nectarines, kiwi fruit, bananas and many berries, is not well-absorbed in the small intestine and reaches the large intestine in substantial amounts, where colonic bacteria cleave the rutinose moiety (to free the aglycone quercetin) prior to ring fission, releasing 3,4-dihydroxyphenylacetic acid, which is absorbed into the bloodstream and methylated in the liver to HVA, before excretion in urine along with 3-hydroxyphenylacetic acid (Figure 1) [1, 17]. Urinary HVA may be elevated up to 2-3 days after dietary exposure due to the delayed involvement of colonic bacterial conversion. There is, thus, a potential for a range of common foods rich in dietary quercetin compounds to contribute to false-positive HVA tests in patients being assessed for possible phaeochromocytoma or related tumours.

Using a randomized crossover dietary intervention, this study aims to establish whether a diet rich in flavonols, especially rutin and quercetin, is likely to raise HVA concentrations beyond the normal threshold (40 µmoles/ 24hours) and contribute to the occurrence of false-positives. Urinary HVA measurement were performed using a routine HPLC method as well as a GC-MS method to exclude possible interference by extraneous compounds [4].

## MATERIAL AND METHODS:

### Materials

All chemicals and solvents were purchased from Sigma Aldrich (Poole, Dorset, UK), except for ethyl acetate (HPLC grade), which was purchased from Rathburn Chemicals (Walkerburn, Pebbles, Scotland, UK).

### High-HVA urinary samples

Urine samples displaying high HVA, obtained during routine investigations from both adults and infants either bearing a catecholamine-producing tumour or false-positive (when no medical explanation was found justifying high HVA), were collected at Crosshouse hospital (-20ºC), analyzed by both HPLC and GC-MS as described below.

### Dietary Intervention and sample collection

Fifteen healthy males and females, aged 21-42 years, not pregnant and not receiving L-dopa or other drugs known to interfere with the measurement of HVA were recruited by local advertisement in Glasgow and completed the study. In parallel, two patients having previously displayed elevated HVA without any tumour being detected also agreed to take part in the study. Ethical permission to carry out the study was granted by the local NHS ethics committee and informed consent was obtained from all volunteers. After baseline measurement (weight, height, waist circumference and blood pressure), the volunteers were randomized in a crossover design to be allocated to be under either a three-day low or high flavonol diet separated by at least 7 days washout. During the low-flavonol diet, volunteers avoided consumption of fruits, vegetables, whole-wheat products, spices, chocolate, tea, coffee, wine and juices. During the high-flavonol diet, volunteers followed their usual diet, supplemented with foods rich in flavonols compounds such as rutin and quercetin (intake equivalent to at least 3 portions per day). The foods provided were vine tomatoes, red onion, yellow onions, tomato soups, onion soups, plums, strawberries, tomato sauces and purple grape juice, purchased from a local supermarket (Morrison plc, Anniesland, Glasgow, UK). Compliance was

validated using a retrospective dietary intake assessment. Volunteers collected 24 hour urine samples, including the second urine passed on day three of each diet and all subsequent fractions, including the first urine passed on day four. Urine was kept cool at all time. Total urine volumes were recorded, urine samples aliquoted and stored at -80ºC until analysis.

**Urinary catecholamine metabolite measurements by HPLC**

HVA measurements were performed at Crosshouse Hospital, Kilmarnock, Scotland for each volunteer after each study period, using an automated HPLC kit method (Bio-Rad Laboratories, Hemel Hempstead, UK) on Gilson equipment that included the ASPEC automated sample preparation and injection unit (Gilson Medical Electronics Inc., Middleton, WI) and an ESA Coulochem II coulometric detector as described by Davidson et al. [8].

**Phenolic acids extraction, derivatisation and analysis by GC-MS**

Phenolic acid extraction, derivatisation and analysis were adapted from Grun *et al* [18]. Internal standard (2,4,5-trimethoxycinnamic acid, 1 mg/ml, 30 µl) was added to urine aliquots and calibration samples (0.5 ml) prior to vortexing. Phenolic acids were precipitated by adding 60 µl of 1M HCl to the samples, which were vortexed and placed at 4ºC for 10 minutes. Anhydrous ethyl acetate (1.5 ml) was added to the samples, which were vortexed for 30 seconds and centrifuged at 2700 rpm for 10 minutes. The upper organic layer was transferred to an amber glass vial placed in a 37ºC aluminum block and dried under a gentle flow of nitrogen. The extraction was repeated once more and the upper organic layers were pooled for each sample. Dichloromethane (200 µl) was added to rinse the walls of the vials and dried under nitrogen at 37ºC. Derivatisation reagent (N,o-Bis (Trimethylsilyl) trifluoroacetamide (BSTFA) + 10% trimethylchlorosilane (TMCS, 50 µl) was added to the vials, the headspace was flushed with a gentle flow of nitrogen prior to capping the vial. Samples were derivatised at 65ºC for 4 hours. Anhydrous hexane (99%, 350 µl) was added to each sample, prior to analysis by gas chromatography-mass spectrometry. A set of standard

calibration solutions, containing phenolic acids ranging from 1 µg to 20 µg/ml, was extracted and analyzed alongside all urine samples.

Phenolic acids were analyzed on a Trace GC interfaced to a DSQ mass spectrometer equipped with a split/splitless injector and an AI3000 autosampler (Thermo Fisher, Hemel Hampsted, UK). Samples (1µl) were injected in split mode, with a 1:25 split ratio. The inlet temperature was maintained at 220ºC. The oven was programmed from 40ºC (held 0.1 min) to 160ºC at 20ºC/min, to 200ºC at 1.5ºC/min, to 250ºC at 10C/min to a final temperature of 300ºC at 40ºC/min, held for 5 min. The transfer lined was maintained at 310ºC. The carrier gas flow (helium) was constant, at 1.2 ml/min. Acquisition was performed in positive EI (electron ionization) mode in full scan (m/z 50-600) with an ionization energy of 70 eV, from 6 to 35 minutes. Acquisition and analysis of GC-MS data was performed on Xcalibur version 2. Identification of phenolic acids was achieved by comparison with the retention times and mass spectra of authentic standards. All samples were analyzed batch-wise, with grouped analysis of samples from same individuals.

**Statistical analysis**

Normality of the data sets was assessed using the Shapiro-Wilk test in SPSS. Data were expressed as median values (inter-quartile range), and analyzed using the Wilcoxon matched pairs test (SPSS). The nonparametric regression procedure of Passing and Bablok [19] was used for comparison of the GC-MS and the HPLC procedures.

**RESULTS:**

**Comparison of HVA measurements by HPLC and GC-MS**

Urinary HVA (n=55) ranged from 13 to 605 µmole/L when analyzed by HPLC, and 3.4 to 541.4 µmole/L when analyzed by GC-MS. Overall, there was a good agreement between the results obtained with both methods (Figure 2, $R^2$=0.97, p<0.001), with a modest constant bias: the Passing and Bablok regression line was: GC-MS assay = 0.93 HPLC assay – 7.97 µmole/L, standard error of the estimate $S_{y.x}$ =15.92.

**Interference of flavonol intake on excretion of HVA and related phenolic acids**

Urinary HVA concentrations were significantly higher after the 3-day high-flavonol diet rich in rutin and quercetin (17 µmoles/24h, interquartile range (IQR) 8) compared to the HVA concentrations excreted following the low-flavonol diet (12 µmoles/24h, IQR 7) (Figure 3A, p<0.001). A similar effect was observed for 3-hydroxyphenylacetic acid, with 38 µmoles/24h, IQR 49 and 5 µmoles/24h, IQR 5, after high and low flavonol diets, respectively (Figure 3B, p<0.001), and 3,4-dihydroxyphenylacetic acid, with 6 µmoles/24h, IQR 6 and 3 µmoles/24h, IQR 1, after high and low flavonol diets, respectively (Figure 3C, p<0.001). Inter-individual variability was large, for all urinary phenolic acids, including HVA, measured during the high-flavonol diet.

Urinary HVA remained within the normal range (<40 µmoles/24h) for all volunteers during the low-flavonol diet. After the high flavonol diet, urinary HVA concentrations using the routine HPLC method exceeded the threshold 'upper limit of normal' value (40 µmoles/24h) for three out of fifteen volunteers. This pattern was also observed using GC-MS, the highest value for one of the volunteers by this method being 51 µmole/24 h.

Given the possibility that some subjects may respond more excessively to high-flavonol diets with high urinary HVA excretion and that these subjects might be over-represented amongst the "false-positives" results in routine investigations, two former "false-positive" patients completed the

dietary intervention. While their urinary HVA excretions had been 43 and 44 µmoles/24 h at routine diagnostic screening, these false-positive patient subsequently did not show similarly high HVA concentrations on either low (6 and 13 µmoles/24 h, respectively) or high flavonol diet (17 and 23 µmoles/24 h, respectively) (Figure 3A).

## DISCUSSION

We have shown good correlation between the HPLC method used routinely to screen urine samples for HVA excretion and a research based GC-MS method. The elevated HVA detected in routine diagnostic tests proved to be HVA, and not a co-eluting compound.

The dietary intervention study required healthy volunteers to follow a diet rich in the flavonols rutin and quercetin, found in ordinary foods [20-22]. Intake of flavonol-rich foods leads to a very clear, statistically significant rise in urinary HVA, accompanied by a significant rise in other urinary phenolic acids (3-hydroxyphenylacetic acid and 3,4-dihydrophenylacetic acid), corresponding to colonic degradation products from the flavonoids rutin and quercetin [17]. Using the routine laboratory HPLC method, three volunteers exceeded the urinary HVA threshold upper limit of normal value. This result was confirmed by similar values using GC-MS. There was, however, a range in the responses, and the large inter-individual variability, with no change in urinary HVA, or in urinary excretion of other metabolites in several subjects post flavonol-rich diet. This variation in response could reflect either dietary compliance during the study, variation in total flavonol intake or alternatively inter-individual differences in the metabolism and excretion of quercetin metabolites. The formerly identified false-positive patients taking part in the dietary intervention study displayed elevated HVA concentrations following the high-flavonol diet. However, the HVA concentrations remained within the normal range, with no exaggerated response to flavonol intake.

Several studies have investigated the influence of a catecholamine-rich diet on the excretion of both urinary and plasma catecholamines and catecholamine metabolites [23-26], with catecholamine-rich foods interfering in particular with plama and urinary 3-O-methylated metabolites of catecholamines, as well as deconjugated normetanephrines [24]. Other potential dietary sources of HVA elevation include tyramine, a monoamine found in a range of food stuff including processed meats and cheese, avocado and bananas, which can be metabolized to dopamine by the hepatic

microsome [27], and hydroxytyrosol, found in olive oil and red wine, a dopamine precursor [28, 29]. Our study, a randomized cross-over intervention, adds to this body of evidence, showing that a normal diet, rich in flavonols, such as the quercetin and rutin contained in common foodstuff (e.g. tomatoes and onions), do indeed significantly increase urinary HVA excretion in healthy volunteers, and therefore interfere with the HVA assay used during diagnosis of catecholamine-producing tumours. A single previous intervention on infants revealed that a diet rich in catecholamine and phenolic acids did not significantly interfere with either catecholamine or their metabolites (including HVA); however, the small sample size (n=6) and analytical techniques used at the time may explain for the lack of effect of the test diet [26].

The diagnosis of catecholamine-producing tumours depends on the measurement of elevated levels of catecholamines and/or their metabolites, with free metanephrines emerging as a robust marker [30], relatively insensitive to dietary variations, but however sensitive to blood sampling position [24]. However, biochemical testing of catecholamine-secreting tumours still routinely involves urinary HVA measurement [31, 32], which we showed to be susceptible to variations linked to dietary interferences. In light of our results, it is therefore appropriate to advise patients to restrict their diets prior to taking the test, avoiding flavonol-rich foodstuff, as listed in Table 1, for a period of time lasting at least three days. The three day flavonol free diet should allow for urinary excretion of phenolic acid metabolites (HVA) of dietary flavonols (50.5% of the dose excreted in urine 48h post-ingestion) [33]. Alternatively, measurements not subject to dietary interference, such as free metanephrines, should be favoured for the diagnosis of catecholamine-secreting tumours [24].

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