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Ultrasensitive assay for three polyphenols (catechin, quercetin and resveratrol) and their conjugates in biological fluids utilizing gas chromatography with mass selective detection

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Abstract

The concentrations of three polyphenols ((+)-catechin, quercetin and *trans*-resveratrol) in blood serum, plasma and urine, as well as whole blood, have been measured after their oral and intragastric administration, respectively, to humans and rats. The method developed for this purpose utilized ethyl acetate extraction of 100 μl samples and their derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA) followed by gas-chromatographic analysis on a DB-5 column followed by mass selective detection employing two target ions and one qualifier ion for each compound. Total run time was 17 min with excellent resolution and linearity. The limits of detection (LOD) and quantitation (LOQ) were an order of magnitude less than for any previously published method, being 0.01 μg/l and 0.1 μg/l, respectively, for all compounds. Recovery at 1 μg/l and 10 μg/l was >80% in all instances but one, and was >90% in 50%. Imprecision was acceptable at 0.25 and 1.0 μg/l, concentrations below the LOQ of previous methods. Aglycones released from conjugates after hydrolysis were easily measurable. Optimal conditions for hydrolysis were established. After oral administration of the three polyphenols to humans, their conjugates vastly exceeded the concentrations of the aglycones in both plasma and urine. Concentrations peaked within 0.5–1.0 h in plasma and within 8 h in urine. During the first 24 h, 5.1% of the (+)-catechin and 24.6% of the *trans*-resveratrol given were recovered in the urine (free plus conjugated). This method can be proposed as the method of choice to assay these polyphenols and their conjugates in biological fluids.

Keywords: Polyphenols; Catechin; Quercetin; Resveratrol

1. Introduction

Population-based analyses and similar epidemiological studies have overwhelmingly supported the notion that moderate alcohol consumption lowers the risks of mortality, hospitalization and early clinical symptoms of coronary artery disease (CAD) compared with the risks attributable to abstinence from alcohol [1–5]. A high percentage of these benefits appear to be direct biochemical consequences of alcohol itself, such as increase in high-

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density lipoproteins (HDL [6]), inhibition of platelet aggregation [7], and enhanced fibrinolysis [8].

Because of its high content of polyphenolic antioxidants (see [9] and [10] for review), red wine has been proposed to be the most advantageous of all alcoholic beverages in lowering CAD risk [11,12], but a major meta-analysis of 25 epidemiologic surveys focusing upon this issue has failed to support this hypothesis [13]. One major concern is whether those polyphenols present in red wine and known to have powerful in vitro biochemical functions likely to attenuate the development and progression of CAD, such as *trans*-resveratrol, (+)-catechin and quercetin, are actually absorbed sufficiently to reach biologically meaningful concentrations in blood and tissues. This issue is of relevance beyond the focus of CAD, since these polyphenols demonstrate potent antimutagenic, anticancer and anti-inflammatory properties as well as being effective free-radical scavengers (see [10] for review).

Before questions about bioavailability can be addressed, ultrasensitive methods are necessary to assay the low concentrations of polyphenols likely to be present in blood and urine assuming their relatively efficient absorption from wine, in which they are present in micromolar amounts. Several decades ago, the absorption and urinary excretion of (+)-catechin in human subjects was indirectly demonstrated by measurement of total phenols [14] and tracer studies with [^{14}C](+)-catechin [15]. Absorption exceeded 50% of the dose given; blood levels peaked around 3 h and persisted for at least 12 h. More recent investigations using direct high-performance liquid chromatography (HPLC) analyses were consistent with these observations [16,17]. Other methods utilizing UV [18] and fluorescence [19] detection following HPLC have also been presented.

Gugler et al. [20] were unable to detect quercetin in the urine of human subjects after a large oral dose (4 g), but several investigators have subsequently demonstrated the absorption of quercetin in humans [21–24] and in rats [25,26] by means of assays in plasma and urine. A high proportion of absorbed quercetin was present as glucuronides, glycosides and sulfates [27–30].

Several papers have described the measurement of *trans*-resveratrol when added exogenously to human serum [31–33]. Bertelli and colleagues [34,35] were the first to demonstrate the absorption of *trans*-resveratrol into rat blood and its excretion in the urine. Juan et al. [36] and Zhu et al. [37] subsequently described methods that also were able to quantitate *trans*-resveratrol in rat blood after oral ingestion. Very recently we demonstrated for the first time the absorption of *trans*-resveratrol in humans and documented its appearance in blood and urine [38]. The method that we developed [39] permitted the simultaneous determination of (+)-catechin and quercetin as well as *trans*-resveratrol. It has since been improved to provide a 10-fold increase in sensitivity. This paper describes the new method and compares its performance characteristics with those of others used for the independent assay of these three polyphenols in serum or plasma.

2. Experimental

2.1. Chemicals and reagents

Pure authentic standards of (+)-catechin (Cat. No. 86181-2), quercetin (Cat. No. 17196-4), and *trans*-resveratrol (Cat. No. R-5010) as well as fisetin (Cat. No. F50-5) were purchased from Sigma–Aldrich Canada, Mississauga, Ontario, Canada. Their structures are shown in Fig. 1. Stock concentrated standard solutions of each (1 g/l) in acetonitrile were stored at 4°C under nitrogen, wrapped in foil, and

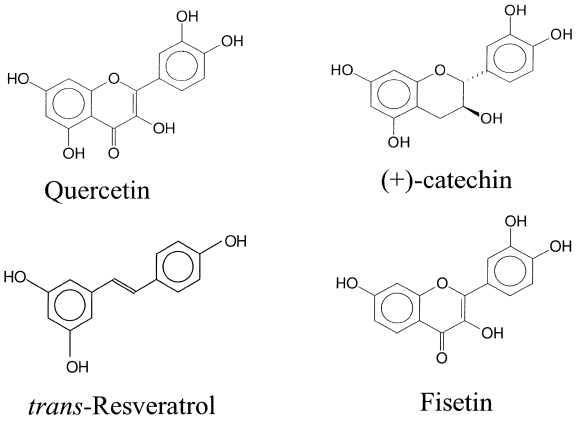

Fig. 1. Structures of the three polyphenols assayed and internal standard (fisetin).

were stable for 6 months. β-glucuronidase from *Helix pomatia*, Type H-5 was purchased as a lyophilized powder from Sigma. β-glucuronidase activity was 477 600 U/g and sulfatase activity was 15 000–40 000 U/g according to the supplier's specifications. It was stored under nitrogen at −20°C. A working solution was freshly made each day comprising 40 mg of powder dissolved in 1 ml of 0.58 *M* acetic acid. 25 μl of this solution (i.e. 1 mg of enzyme equivalent to 478 U of β-glucuronidase and 15–40 U of sulfatase) was added to 100 μl of each plasma or urine sample to hydrolyse the conjugated polyphenols. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Cat. No. 3-3084) was obtained from Supelco, Bellefonte, PA, USA. The DB-5 column (15 m long, 0.25 mm I.D., and 0.25 μm film thickness) was from J&W, Folsom, CA, USA. The gas was purchased from Praxair, Mississauga, Ontario, Canada. All other solvents and reagents were from Caledon Laboratories, Georgetown, Ontario, Canada.

2.2. Biological samples

Blood samples were taken by cubital venipuncture from 10 human volunteers under various conditions, including after an overnight fast and at various time intervals after the oral ingestion of the pure standards dissolved in 10% (v/v) ethanol, or after various beverages containing these polyphenols such as white wine (Lindemans Chardonnay, Australia), grape juice (Sunfresh, Toronto, Canada), or vegetable cocktail (Campbell Soup Company, Toronto, Canada). The approximate polyphenol concentrations of these beverages are given in Table 1. In most cases, the whole blood was allowed to clot and the serum was separated after centrifugation at 2000 *g* for 10 min at 4°C. In some instances, whole blood was obtained by collecting the samples into tubes containing disodium EDTA (1.5 mg/ml). Where conjugated polyphenols were measured, the blood was collected into tubes containing heparin sodium and plasma was separated by centrifugation, since EDTA inhibits the enzymes used to hydrolyse the conjugates by chelating Ca^{2+}. Urine samples were also collected from these volunteers under the same dietary conditions as the blood samples.

Serum and whole blood were collected from male Wistar rats (Charles River, Quebec, Canada) by cardiac puncture after an overnight fast or after the administration by gavage of pure standards in 10% (v/v) ethanol, white wine, or vegetable cocktail. Urine was also collected from these animals by placing them in metabolic cages. To measure recovery, known amounts of the pure standards were added to serum and urine matrices; after thorough mixing and standing at 4°C for 1 h in the dark, multiple assays were performed on the individual samples.

All procedures employed were subject to prior approval by the human and animal experimentation committees of the University of Toronto.

2.3. Sample preparation

One hundred μl (serum or urine) was extracted twice with 0.8 ml followed by 0.5 ml of ethyl acetate by vortexing for 1 min in a 1 ml vial. The second extraction removed about 5–10% of the first; a third extraction (0.5 ml ethyl acetate) removed <1% of the first, and two were considered adequate. After centrifuging the mixture for 5 min at 3500 *g*, the top layers comprising ethyl acetate were removed, pooled and evaporated to dryness under nitrogen. One hundred μl of ethyl acetate previously filtered

Table 1
Concentrations of three polyphenols (free and conjugated) in beverages employed in this study[a]

Polyphenols	Grape juice		White wine		Vegetable cocktail	
	Free	Conjugated	Free	Conjugated	Free	Conjugated
(+)-Catechin	5081	5179	920	1010	163	215
Quercetin	390	1662	176	202	1227	2251
trans-Resveratrol	27	56	85	91	5.6	8.0

[a] Data as average of two independent samples (μg/l).

through anhydrous Na_2SO_4 and 100 μl of BSTFA were added to the dried residue; the mixture was vortexed for 30 s and heated for 1 h at 70°C.

2.4. Enzymatic hydrolysis of conjugates

To 0.1 ml of sample (urine or serum), 25 μl of working enzyme solution representing 1 mg of original enzyme was added. The mixture was vortexed, incubated at 37°C for 2 h, and extracted successively with 0.8 ml and 0.5 ml of ethyl acetate; the latter phases were separated by centrifugation, pooled, and analyzed by derivatization followed by GC–MS as described above. Fisetin may be added at 5 μg/l as internal standard if desired by spiking 20 μl of 50 μg/l fisetin standard diluted in de-ionized water into the sample, before enzyme hydrolysis and extraction. Preliminary experiments established these conditions as optimal for hydrolysis. With respect to time and temperature of incubation, resveratrol conjugates in plasma showed a doubling of reaction rate at all time-points with plasma and urine at 37°C compared with room temperature (20°C). Plasma and urine (+)-catechin showed no temperature dependence, but the concentrations were <10% those of *trans*-resveratrol. At both temperatures, the amount of aglycone released from plasma and urine increased up to 2 h but not beyond. Adding various amounts of enzyme from 0.1 to 1.0 mg per assay increased the rate of hydrolysis, but between 0.5 mg and 1.0 mg per assay the amount of aglycone released rose by only 8% for resveratrol in serum and 15% in urine at comparable levels of conjugates. Findings for (+)-catechin and quercetin were similar.

2.5. Gas chromatographic analysis

The derivatized sample was analyzed by GC–MS using a 6890 GC interfaced to a 5973 MSD, both from Hewlett–Packard, Mississauga, Ontario, Canada. One μl was injected on to the DB-5 MS column. The injector and detector were both set at 280°C. The temperature program comprised two phases: initially the temperature was set at 120°C for 2 min. It was then ramped to 300°C at a rate of 20°C/min and held for 6 min. The total run time was 17.0 min. Ultra-high purity helium with in-line Supelpure moisture trap and hydrocarbon trap (Supelco Canada, Mississauga, Ontario, Canada) was used as a carrier gas. The carrier gas-line was set at 48 p.s.i., column head pressure at 10.9 p.s.i. and total flow at 3.7 ml/min.

The injector was at splitless mode. The elution time and SIM parameters (one target ion and two qualifying ions, dwell time per ion 100 ms) for each compound are presented in Table 2. Typical chromatograms for human serum and urine are shown in Fig. 2. Comparable resolution was achieved for rat serum and urine, as well as whole blood from both species (not shown).

2.6. Calibration and quality control

The peak abundance of the unknown samples was compared with those of authentic pure standards for purposes of quantitation. Six dilutions in an ethyl acetate extract of the matrix (serum or urine) in the same proportion as the samples covering the range 0–20 μg/l for serum and 0–200 μg/l for urine were prepared from each of the concentrated stock standards. They were stable for 2 weeks at 4°C under

Table 2
Analytical characteristics of the present method

Characteristic	(+)-Catechin	Quercetin	*trans*-Resveratrol	Fisetin
Mass spectral features				
Target ion (amu)	369	647	444	471
Qualifier ions (amu)	368, 370	648, 649	445, 446	399, 560
Retention time (min)	8.63	9.61	8.04	9.38
LOD (μg/l)	0.01	0.01	0.01	–
LOQ (μg/l)	0.1	0.1	0.1	–

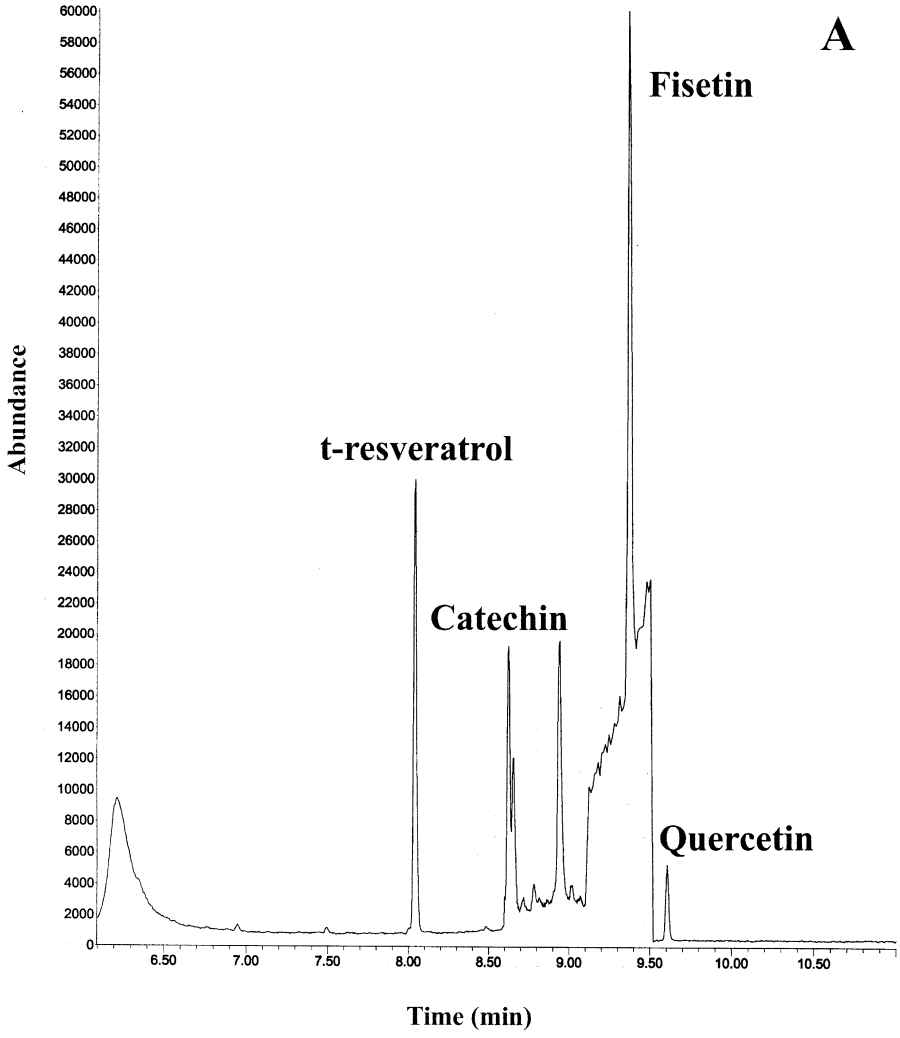

Fig. 2. Chromatograms of three polyphenols and internal standard by selective ion monitoring utilizing the ions listed in Table 2. (A) 1 μg/l of each compound in human serum; (B) 1 μg/l of each compound in human urine.

nitrogen and wrapped in foil. A calibration curve with four assays at each concentration was prepared each week; limits for one SD and two SD were established. At the beginning and end of each working day, one high and one low standard were analyzed. Criteria for acceptability of that batch required that not more than three of the four samples were above or below the calibration line; moreover, it was not acceptable that more than two values lay outside the limit of one SD on either side of the line, and none were permitted beyond the two SD limits. A fresh calibration curve with newly defined SDs was constructed when the above criteria were not met, and the GC–MS analysis of that batch was repeated. Calibration curves with virtually identical slopes and goodness of fit were obtained using the raw matrices but stability of the standards was less than that of the ethyl acetate extracts that seemed to adequately reflect the matrix effects. The use of fisetin is not essential. Results for (+)-catechin and *trans*-resveratrol were not significantly different in its absence or presence, whereas with quercetin the

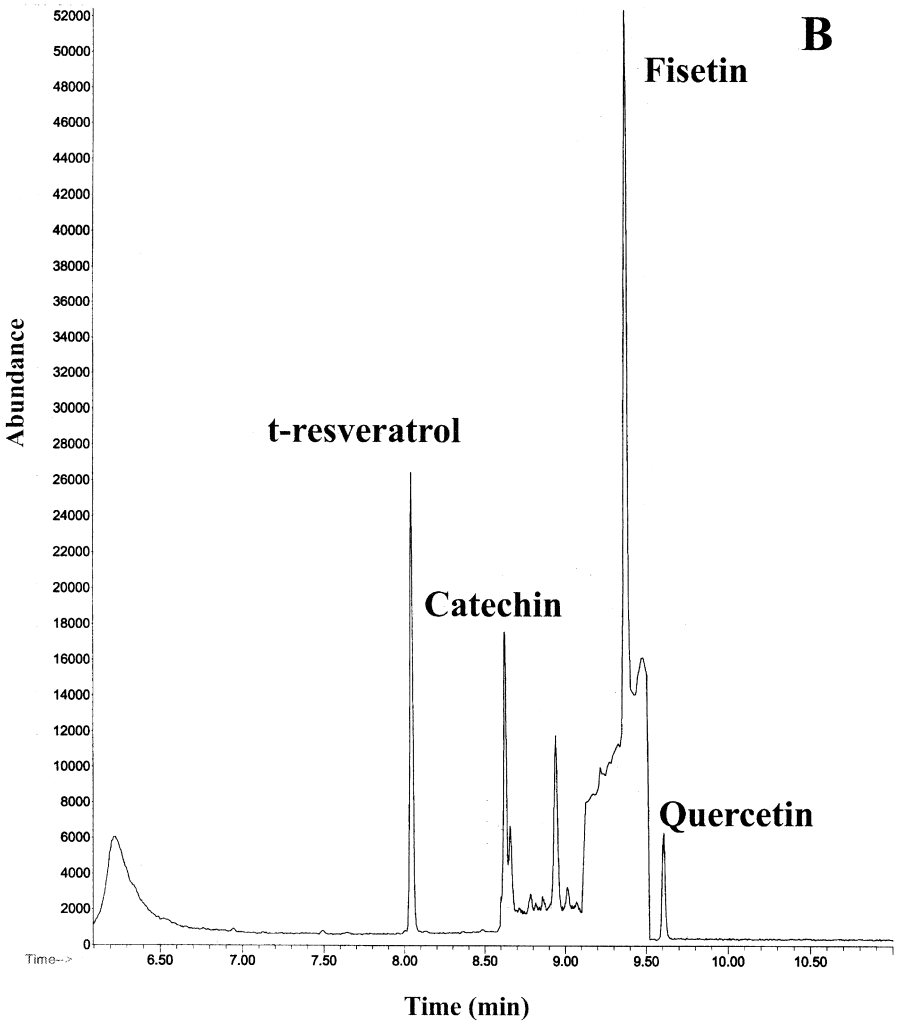

Fig. 2. (*continued*)

uncorrected values were on average 5% lower, a difference that most laboratories can tolerate without the need for internal standardization.

3. Results

3.1. Separation of polyphenols

Excellent resolution was obtained for all three polyphenols in all biological fluids sampled. Fig. 2 demonstrates the resolution obtained from human serum and urine simultaneously spiked with (+)-catechin, quercetin and *trans*-resveratrol. The retention times, listed in Table 2, allowed completion of each run within 17.0 min.

3.2. Method sensitivity

Based upon standard criteria [40], the limits of detection (LOD), defined as the lowest concentration to exceed the mean baseline value by >3 SD, and

the limits of quantitation (LOQ), defined as the lowest concentration of each compound that gave a signal-to-noise ratio of 10 were determined for standards and for serum and urine from rats and humans by spiking each matrix with 100 μg/l of each compound and analyzing each sample 10 times. For all matrices, LOD did not exceed 0.01 μg/l and LOQ did not surpass 0.1 μg/l for any of the three compounds (Table 2).

3.3. Linearity

Excellent linearity was obtained for all three compounds in all matrices. Sufficient of each polyphenol was added independently to each matrix to generate six different concentrations over the range 1–2500 μg/l and quadruplicate analyses were carried out on each concentration. The quantitative parameters based on correlation and regression analyses are listed in Table 3. The closeness of fit is documented by the fact that the lowest *r*-value recorded was 0.998 (*trans*-resveratrol in human and rat urine). As demonstrated by the slopes, the method provided the greatest sensitivity for *trans*-resveratrol in all matrices except rat serum. The *y*-intercepts did not deviate significantly from zero apart from (+)-catechin and quercetin in human urine, and *trans*-resveratrol in rat urine. In the case of the former matrix, the intercepts may be attributable to the endogenous presence of these polyphenols, but this explanation does not seem to be tenable for *trans*-resveratrol in rat urine.

3.4. Recovery

Table 4 presents data on recovery of the three polyphenols from the four matrices as mean and relative standard deviation (RSD) of six independent assays. In most matrices, the recovery of *trans*-resveratrol was superior to that of the other two polyphenols, six of the values being >90%, and the lowest being 89.3% (in human urine). The recovery of quercetin was the poorest of the three, the values being >90% in only two matrices, and as low as 79.6% in rat urine. Further, the variance in recovery for quercetin was greater than that of the other two, an exception being human serum at the 1 μg/l concentration.

Table 3
Linearity of polyphenol assays in blood serum and urine of humans and rats evaluated by correlation and regression analysis [41]

Matrix and compound	Correlation (*r*) coefficient	Slope (*R*) (mv per μg)	*y*-Intercept (% deviation from zero)
Human serum			
(+)-Catechin	0.999	1.48×10^5	1.2
Quercetin	0.999	3.16×10^5	1.4
trans-Resveratrol	0.999	3.38×10^5	2.4
Human urine			
(+)-Catechin	0.999	1.73×10^5	3.5[a]
Quercetin	0.998	2.27×10^5	4.1[a]
trans-Resveratrol	0.999	2.30×10^5	2.2
Rat serum			
(+)-Catechin	0.999	1.09×10^5	2.3
Quercetin	0.999	4.59×10^4	0.3
trans-Resveratrol	0.998	9.65×10^4	1.4
Rat urine			
(+)-Catechin	0.999	5.31×10^4	1.8
Quercetin	0.999	7.02×10^4	1.1
trans-Resveratrol	0.998	1.12×10^5	3.6[a]

[a] Significantly different from zero ($P<0.05$) as evaluated by the Pearson test utilizing the derived SE [41].

Table 4
Recovery of three polyphenols added to serum and urine of rats and humans at two concentrations[a]

Compound and species	Conc. (μg/l)	Serum	Urine
		Mean recovery (%)±RSD (%)	
Human			
(+)-Catechin	10 μg/l	87.3±5.1	86.8±7.5
Quercetin	10 μg/l	92.4±13.8	81.0±12.2
trans-Resveratrol	10 μg/l	94.3±5.9	89.3±6.9
(+)-Catechin	1 μg/l	90.7±6.6	87.7±10.2
Quercetin	1 μg/l	84.6±5.1	91.4±15.6
trans-Resveratrol	1 μg/l	93.9±5.9	95.7±11.7
Rat			
(+)-Catechin	10 μg/l	92.3±8.2	93.9±8.2
Quercetin	10 μg/l	89.2±16.6	84.5±13.4
trans-Resveratrol	10 μg/l	91.7±3.9	94.6±10.1
(+)-Catechin	1 μg/l	95.5±13.5	84.4±12.5
Quercetin	1 μg/l	81.8±19.1	79.6±18.5
trans-Resveratrol	1 μg/l	89.6±6.5	93.2±11.8

[a] All data are means of six independent assays.

Table 5
Imprecision of three polyphenol assays as RSD (%) in blood serum and urine of rats and humans at three concentrations[a]

Compound and matrix	Approximate concentration		
	0.25 μg/l	1.0 μg/l	10 μg/l
Human serum			
(+)-Catechin	4.2	7.8	1.9
Quercetin	5.8	9.1	3.9
trans-Resveratrol	7.1	3.8	1.1
Human urine			
(+)-Catechin	13.2	6.3	5.5
Quercetin	8.6	4.9	8.4
trans-Resveratrol	6.2	2.4	3.4
Rat serum			
(+)-Catechin	5.1	5.4	6.8
Quercetin	13.0	6.1	3.2
trans-Resveratrol	5.9	6.7	5.7
Rat urine			
(+)-Catechin	14.3	8.1	3.8
Quercetin	6.9	4.9	2.3
trans-Resveratrol	2.9	3.5	2.1

[a] Data are RSD based upon six replicate analyses of the same samples spiked simultaneously with all three polyphenols approximating the stated concentration (±15%).

3.5. Analytical imprecision

This was evaluated in all matrices at three concentrations ranging from 0.25 to 10 μg/l (Table 5). Even at the lowest concentration, the imprecision for *trans*-resveratrol was acceptable, but for (+)-catechin in two matrices and for quercetin in rat serum, the RSD was >13%. At 1.0 μg/l, all RSD values were <9.2%, whereas at 10 μg/l all but four were <3.9%.

3.6. Application of method to human subjects

Volunteers were given 25 mg of (+)-catechin, or *trans*-resveratrol or 10 mg of quercetin (previously dissolved in 10 ml of 95% [v/v] ethanol) orally in 120 ml of white wine, after collecting zero-time samples of plasma and urine. Blood samples were taken for the first 4 h and urine samples were collected at various times over the next 24 h. This study is ongoing, but preliminary data are presented in Tables 6 and 7 to demonstrate that both free and conjugated polyphenols are readily measured by this method, even in basal samples taken after abstinence of at least 24 h from foods in which they are known to be present. Over the 24-h collection period, 5.1% of the (+)-catechin, 18.5% of the quercetin, and 24.6% of the *trans*-resveratrol were recovered in the urine as the total of free and conjugated polyphenols. Plasma concentrations of free *trans*-resveratrol were almost an order of magnitude higher than those of (+)-catechin at all time-periods. The former peaked at 30 min and had returned to baseline by 2 h. The latter was around 2.4-fold the basal concentration from 0.5 to 2 h but showed a decline at 4 h. For *trans*-resveratrol, the plasma concentration of conjugates was 30–50-fold higher than the free and also peaked at 30 min, and was below baseline by 2 h. With (+)-catechin, the basal concentration of conjugates was less than the free, peaked at 30-min, started to decline, but was still 30-fold the basal concentration at 4 h. Again, the (+)-catechin conjugate concentrations at all times were at least an order of magnitude less than those of *trans*-resveratrol and, with the exception of the basal sample, almost an order of magnitude greater than the free. The concentrations of free quercetin in plasma before and after oral administration were higher than those of (+)-catechin and *trans*-resveratrol. A peak at 30 min

Table 6
Concentrations of free and conjugated polyphenols in plasma of subjects given 25 mg of (+)-catechin or *trans*-resveratrol or 10 mg of quercetin in 120 ml of white wine[a]

Time of sample (h)	(+)-Catechin (μg/l)		*trans*-Resveratrol (μg/l)		Quercetin μg/l)	
	Free	Conjugated	Free	Conjugated	Free	Conjugated
0	0.35	2.7	4.6	4.7	22.2	43.9
0.5	0.94	16.1	7.1	338	33.5	117.6
1	0.82	14.5	5.9	215	15.7	63.4
2	0.99	8.5	3.4	95	10.5	25.8
4	0.65	5.7	3.1	51	7.5	23.0

[a] All assays in duplicate.

was followed by a decline to below baseline values by 1 h. The concentrations of plasma conjugates behaved in a parallel fashion, were intermediate between those of (+)-catechin and *trans*-resveratrol, and represented a smaller increment over the free quercetin concentrations than was the case with the other two polyphenols.

Six urine samples after oral consumption of the polyphenols were obtained at approximately 4-h intervals over the first 24 h, as well as a basal sample. Free (+)-catechin rose to a peak in the second sample and was back to the baseline value by the fourth sample and continued to fall further. The conjugates peaked in the second sample and were below basal concentrations in the last two. For *trans*-resveratrol, both free and conjugated, the peak concentrations occurred in the second sample but had not returned to the baseline at the end of 24 h. Apart from the basal sample, the concentrations of both free and conjugated *trans*-resveratrol were dramatically higher than those of (+)-catechin, and for both polyphenols the conjugates were 20–30-fold higher than the free. Quercetin was only measured in a basal and composite 24-h urine. The basal and composite ratios of conjugated to free quercetin was lower than for the other two urine polyphenols.

4. Discussion

Up to the present, no method for the assay of multiple polyphenols of different classes in serum, urine and whole blood taken from humans and animals after consumption of these compounds has been described, apart from our earlier paper [39]. This method has now been modified to increase the sensitivity 10-fold, together with much superior resolution, improvements attributable to the utiliza-

Table 7
Concentrations of free and conjugated polyphenols in urine of subjects given 25 mg of (+)-catechin or *trans*-resveratrol or 10 mg of quercetin in 120 ml of white wine[a]

Time of sample (h)	(+)-Catechin (μg/l)		*trans*-Resveratrol (μg/l)		Quercetin (μg/l)	
	Free	Conjugated	Free	Conjugated	Free	Conjugated
0	5.3	69	5.1	64	8.3	45.5
1	14.4	2411	193	6962		
2	20.6	3213	251	7712		
3	6.0	133	119	3559		
4	5.4	211	57	2847		
5	2.6	39	18.6	934		
6	1.8	32	12.8	818	83.1[b]	534.7[b]

[a] Data are means of duplicate assays. Samples were collected at six timed intervals after polyphenol administration approximately 4 h apart. For simplicity, the concentrations have not been corrected for volume or time.
[b] Only a single 24 h composite urine sample was collected in this subject.

tion of the newest HP GC–MSD, the more stable (lower bleed) DB5 MS column, and by refining the GC oven program as well as injector and detector temperatures. Additionally, we have developed an optimized technique for the hydrolysis of conjugates (glucuronides and sulfates) of these polyphenols in human plasma and urine that, for the first time, has permitted the quantitation of their urine output after oral administration of the free polyphenols to humans.

Carando et al. [17] described the measurement of (+)-catechin in human plasma. Extraction with acetonitrile was followed by HPLC on a C_{18} column. The compound of interest eluted after 50 min with a recovery of 85%. Using fluorescence detection, LOD was 5 μg/l and LOQ was 50 μg/l. RSD values ranged from 3.9 to 6.5%.

Hollman and colleagues developed a method to assay quercetin in human plasma and urine employing acid hydrolysis to liberate the aglycone followed by HPLC on a C_{18} column and post-column derivatization with $AlNO_3$ to generate a fluorescent complex [21–23]. Quercetin eluted around 8 min with an LOD of 2 μg/l (LOQ not stated). Recovery was around 88% for plasma and 99% for urine, and RSD was 4% for plasma and 6% for urine. Variants of this method substituting enzymatic (β-glucuronidase/sulfatase) for acid hydrolysis were recently reported by Morrice et al. [42] and by Ader et al. [30]. In the first, quercetin eluted at 13 min but did not seem to be well resolved from the baseline; in human plasma, LOD was 1.7 μg/l, recovery was 97% and RSD was 6.6%. In the second, performed with pig plasma, quercetin eluted around 9 min, LOD was 3 μg/l and recovery was 83%. In the method of Manach et al., the aglycone was generated by enzymatic hydrolysis (β-glucuronidase and sulfatase) followed by HPLC (C_{18} column) and UV detection [25]. The elution time was 6 min and recovery >85%, but no other analytical characteristics were described.

Trans-resveratrol was measured in rat serum and urine after gastric administration of red wine by a method employing an LC–MS system with two HPLC columns in tandem after a lengthy extraction procedure [34]. No performance data were given, apart from a statement that the LOQ is 1 μg/l. Another method to measure *trans*-resveratrol in the serum of rats utilized HPLC on a C_{18} column followed by photodiode array detection in the UV range [36]. The RSD was <3%, but the LOD was 20 μg/l. A similar method was used by Kuhnle et al. [43], who also performed analyses based on LC/MS/MS technology. Both methods were utilized in the determination of resveratrol glucuronide in rat intestinal fluid after enzymatic hydrolysis, but performance characteristics were not described. Finally, both isomers of resveratrol were measured in rat whole blood using HPLC on a C_{18} column followed by electrochemical detection [37]. The total run-time was <10 min; recovery was 63.5%; RSD was 2.5–4.4%; LOD and LOQ were 2 μg/l and 4 μg/l, respectively, with linearity spanning the range 5–1000 μg/l.

Bearing in mind the fact that all three compounds are predominantly present in plasma and urine as glucuronides and sulfates [21,27,30,43–47], the ability to measure these conjugates is a major step towards accurate assessment of the bioavailability of the parent compounds. The absorption by humans of (+)-catechin can be calculated from plasma time-curves recently published by Bell et al. [45]. Absorption of a 35 mg dose approximated 7.9%, and the peak plasma concentrations for free and conjugated forms was around 1 h. Since these authors had earlier demonstrated the 3′ and 4′ *O*-methylation of (+)-catechin and its subsequent conjugation [48,49], this estimate is certainly on the low side. Our own previous experiments in rats using [^{3}H]*trans*-resveratrol and in humans using chemical measurement of free *trans*-resveratrol [38] have yielded results that, in the first model are well above, and in the second model somewhat below the estimates for absorption in the present investigation. Methylation of *trans*-resveratrol has not been described, but this and other metabolic transformations may account for our urinary recovery being less in humans than recovery of the [^{3}H]-label in rats [38]. Hollman and colleagues have estimated that humans absorb 30% of an oral dose of quercetin [22], whereas Ader et al. [30] could only recover 8.6% of an intravenous (IV) dose as free and conjugates in pigs, but the metabolism of this compound is very complex, giving rise to isorahmnetin and kaempferol, among others. Taking these into account, recovery of IV-administered quercetin rose to 17% [30].

The method presented in this report is at least an order-of-magnitude more sensitive for all three con-

stituents than any other previously published. The elution times are satisfactory; recovery was better than that of most methods; imprecision was comparable with that of other authors, but at concentrations well below the LOQ that they were able to achieve. Linearity was excellent, covering the range of concentrations likely to appear physiologically in serum and urine, below the capability of other methods. Glucuronides and sulfate conjugates are easily measured. Finally in addition to its sensitivity, the specificity guaranteed by MS technology is another compelling argument for the adoption of this method.

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