Measurement of Resveratrol, Kaempferol, Quercetin and Isorhamnetin in Human Plasma

Paul Gamache and Ian Acworth
Thermo Fisher Scientific, Chelmsford, MA, USA

Key Words
Metabolites, HPLC-ECD, Flavonoids, Glycosides

Goal

To develop an HPLC-ECD method capable of sensitive detection of isorhamnetin, kaempferol, quercetin and resveratrol in extracts of human plasma

Introduction

There has been a great deal of interest in the potential health benefits of flavonoids and phenolic compounds present in fruits and vegetables. The most widely studied compounds include catechins, flavonols and phytoestrogens. The biological effects of these compounds, as shown by numerous *in vitro* and animal studies, suggest that they may be protective against cancer, cardiovascular, inflammatory and other diseases. Several *in vitro* studies have also suggested pro-oxidant and mutagenic effects. Although there have been great strides in understanding the occurrence, bio-availability and activities of these compounds, relatively little is known of their *in vivo* effects in humans. Analytical methods that are capable of measuring low levels of these compounds and their metabolites in biological tissues are therefore needed.

Several methods based on HPLC with electrochemical detection (ECD) have recently been shown to be useful for these purposes primarily due to its high sensitivity and selectivity for detection of compounds with phenolic substituents.[1–9] The present study used HPLC with coulometric array electrochemical detection.[3,4,6–10] This technique utilizes multiple sensors that can be optimized for more than one chemical class. Easily oxidized compounds can be selectively detected at upstream, low potential sensors, while higher oxidizing compounds respond at downstream higher potential sensors.

Our recent objectives have been to develop a method for analysis of quercetin, its metabolite, isorhamnetin, kaempferol and resveratrol in human plasma (Figure 1). Quercetin and kaempferol are widely distributed flavonols found, typically as glycosides, in many fruits and vegetables. The simultaneous determination of quercetin and kaempferol in plasma may be useful as an indicator of fruit and vegetable consumption.[11] The 3´-O-methylated form of quercetin, isorhamnetin, has been shown to be a principle metabolite in rats.[12] These compounds are believed to exist mainly as sulfo- and/or glucurono-conjugates in plasma. Resveratrol, present in red wine,[13,14] has received particular attention for its potential role in preventing cardiovascular disease.[15–18]

Thermo SCIENTIFIC

Relatively little few studies have addressed the bio-availability and metabolism of resveratrol in humans. Presented here are preliminary analytical performance data (e.g., linearity, limits of detection, precision) and methodological considerations for measurement of these compounds in biological matrices.

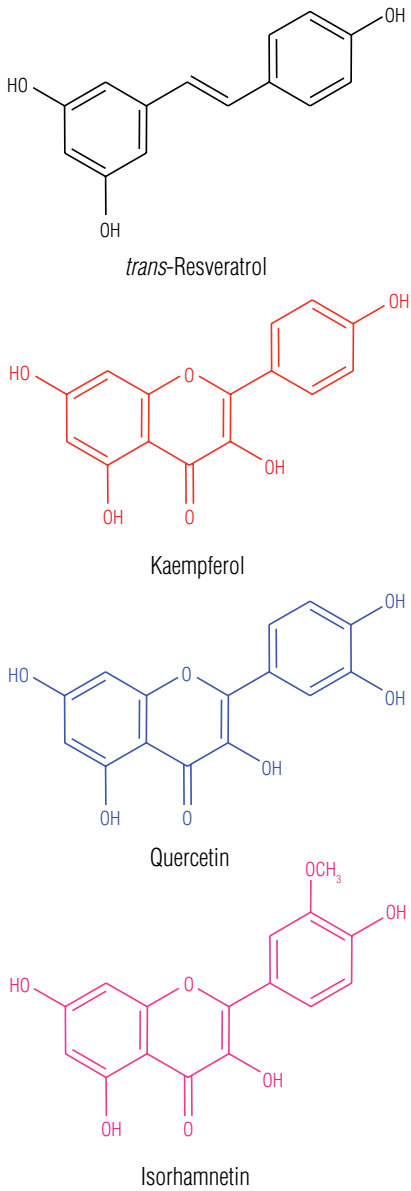

Figure 1. The chemical structures of resveratrol, kaempferol, quercetin, and isorhamnetin.

Materials and Methods

The gradient analytical system consisted of two pumps, an autosampler, a thermostatic chamber and an eight-channel Thermo Scientific™ Dionex™ CoulArray™ Coulometric Array Detector.

LC Conditions	
Column:	C18, 3.0 × 150 mm, 3 µm
Mobile Phase A:	Acetonitrile - water, 10:90 (v/v) containing 75 mM citric acid and 25 mM ammonium acetate
Mobile Phase B:	Acetonitrile - water, 50:50 (v/v) containing 75 mM citric acid and 25 mM ammonium acetate
Gradient:	0–35 min linear from 10% to 80% B; 35–50 min re-equilibration at 10% B
Flow Rate:	0.6 mL/min
Temperature:	Ambient
Injection Volume:	20 µL
Detectors Conditions	
Electrochemical Detector:	Model 5600A, CoulArray
Applied Potentials:	100, 200, 320, 380, 440, 500, 560, and 620 (mV vs Pd)
Cell Cleaning:	All cell potentials were set to 900 mV (vs Pd) for 0.5 min at the beginning of each 5.0 min re-equilibration period to minimize electrode surface adsorption

Sample Preparation

1.0 mL of serum was incubated at 37 °C overnight with 10 µL of 10 µg/mL estriol-3-(β-glucuronide) [internal standard], 0.1 mL of 0.1 M ascorbic acid, 0.25 mL of 1.0 M ammonium acetate buffer, pH 5.0 and 1,000 units of β-glucuronidase from *Helix pomatia*. After addition of 0.1 mL of glacial acetic acid, hydrolysates were washed with 5.0 mL of hexane and extracted with two 3.0 mL volumes of diethyl ether. Combined extracts were evaporated to near dryness under N_2 and the resulting residue dissolved by sonication for 1 min in 0.2 mL of methanol followed by addition of 0.2 mL of water. Samples were then kept at 4 °C for 30 min and centrifuged at 14,000 rpm for 10 min prior to HPLC-ECD.

Detection Linearity, Lower Limit of Detection and Variability

Response linearity was assessed with standards (9 levels, 1.0 ng/mL to 10.0 µg/mL, 20 µL injection volume) diluted in 10% methanol containing 0.01 M ascorbic acid using least squares regression analysis of peak height response vs amount injected. Lower limit of detection (LOD) was estimated from standard mixtures using a signal to noise ratio of 3:1. Intra-assay variability was estimated from 5 replicate analyses of spiked plasma at 50 and 500 ng/mL.

Results and Discussion

Chromatographic and response behavior from a spiked plasma sample is shown in Figure 2. Easily oxidized compounds (quercetin, kaempferol and isorhamnetin) responded predominantly at the first sensor (100 mV) followed by resveratrol at the second coulometric sensor (200 mV). Estriol, the internal standard, which was formed by enzymatic hydrolysis of estriol-3-β-glucuronide responded predominantly on sensor 8 (620 mV). The selectivity was increased for each class of compound since lower oxidizing interfering solutes were oxidatively screened at upstream sensors. Using an array of potentials along the oxidative or reductive curve of each analyte allowed generation of response ratios for each peak. The response ratios obtained between adjacent channels were therefore descriptive of the voltammetric behavior of each analyte. In these studies the ratio data obtained from spiked samples compared well with those from authentic standards thus indicating selective detection.

The described chromatographic and detection conditions are similar to those previously described for analysis of isoflavones and lignans.[4,9] The retention times for daidzein, enterodiol, genistein and enterolactone using these conditions were 16.3, 17,4, 22.0, and 23.3 min, respectively (data not shown). Peak pairs: quercetin/enterodiol, kaempferol/genistein and isorhamnetin/enterolactone were thus poorly resolved chromatographically. Since the isoflavones and lignans oxidize at potentials 200 mV higher than quercetin, kaempferol and isorhamnetin these compounds can be resolved voltammetrically by using a coulometric array detector. Since the sample extraction conditions used herein are also similar to those described for analysis of plasma phytoestrogens,[4,9] it is possible that they may be analyzed concurrently with resveratrol and the flavonols. More importantly, when using a less selective detector, the possibility that the commonly occurring phytoestrogens may interfere with analysis of flavonols must be considered.

Estriol-3-β-glucuronide was used as an internal standard. This approach provided some indication of both the efficiency of enzymatic (glucuronidase) hydrolysis and the efficiency of extraction. The amount added to plasma was several orders of magnitude higher than reported endogenous levels in order to avoid any significant contribution to the internal standard response.

A common problem encountered when analyzing easily oxidized compounds is auto-oxidation, which typically leads to disproportionate loss of sensitivity at low analyte levels. Our previous studies demonstrated that use of a lower pH mobile phase that also incorporated metal-chelating properties minimized auto-oxidation within the chromatographic system. For these reasons, the mobile phase conditions utilized in these studies included an acetate-citrate buffer, pH 3.0. Also, as shown previously,[1,6] the incorporation of ascorbic acid in the hydrolysis mixture improves the stability of easily oxidized catechols.

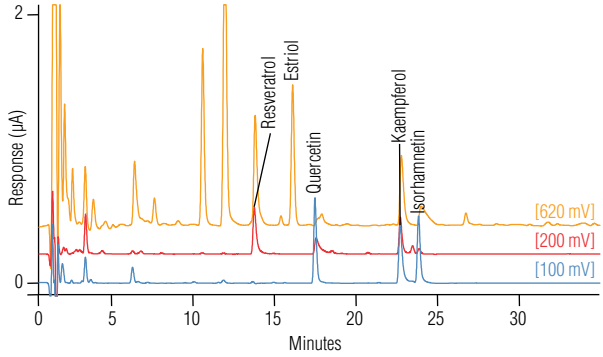

Figure 2. Chromatogram (channels 1, 2, and 8) of extracted human plasma augmented with standard mixture (500 ng of each analyte per mL of plasma).

Linearity data obtained from diluted standards are shown in Table 1. The slope obtained from least squares regression analysis indicates high sensitivity (nA/ng) for all analytes. The response for resveratrol, which required the highest oxidation potential, was proportionally the most linear among these analytes. There was some deviation from linearity for the more easily oxidized compounds (quercetin, kaempferol and isorhamnetin) which may be a result of auto-oxidation. Initial studies of human plasma, augmented with standards over this same range of analyte levels demonstrated high (greater than 90 percent) absolute extraction recovery with a similar deviation from linearity (data not shown). This requires further investigation. Based on a signal to noise ratio of 3:1 the lower limits of detection for standard compounds were approximately 10–20 pg on column for all analytes.

Table 1. Response linearity.

Compound Name	Slope (nA/nA)	Y-Intercept nA	Correlation Coefficient (r)
Resveratrol	46.9	-74.2	0.999
Quercetin	40.0	-102	0.997
Kaempferol	42.4	-117	0.993
Isorhamnetin	26.2	-119	0.964

Figure 3 shows a plasma sample that was augmented with 5 ng of each analyte per mL of plasma prior to extraction. Additional studies are required to determine limits of detection and quantitation in extracted plasma samples.

For replicate injections of un-extracted standards, intra-assay response variability ranged from 0.5 to 1.2% R.S.D. for all analytes (data not shown). However, when analyzing replicates of extracted plasma the variability was much higher as shown in Table 2. Most of the variability therefore comes from the extraction procedure and further work is required.

Conclusion

This preliminary data show that HPLC-coulometric array method is capable of sensitive detection of isorhamnetin, kaempferol, quercetin and resveratrol in extracts of human plasma. Differences in oxidation-reduction properties of these compounds allowed resolution of easily oxidized flavonols from higher oxidizing and provided qualitative peak purity data. On-column oxidation which led to deterioration in response was minimized by using an acetate-citrate based mobile phase. Detector response was precise (<1.2% R.S.D.), and sensitive (10–22 pg LOD). Deviations from linearity and variability in extraction efficiency are believed to result from auto-oxidation of these compounds. Our future efforts will focus on optimization of the extraction and separation conditions to minimize auto-oxidation prior to conducting validation studies and dietary studies with human subjects.

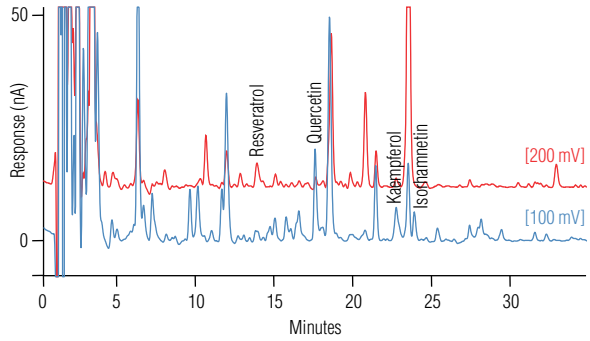

Figure 3. Chromatogram (channels 1 and 2) of extracted human plasma augmented with standard mixture (5 ng of each analyte per mL of plasma).

Table 2. Intra-assay variability.

Compound Name	500 (ng/mL) %RSD	50 (ng/mL) %RSD
Resveratrol	9.3	2.7
Quercetin	9.2	4.4
Kaempferol	13.2	5.5
Isorhamnetin	7.9	8.7

References

1. Erlund, I., Alfthan, G. Siren, H. Ariniemi, K., and Aro A. (1999). Validated method for the quantitation of quercetin from human plasma using high-performance liquid chromatography with electrochemical detection. J. Chromatogr. B, 727, 179–189.
2. Franke, A. A., and Custer, L. J. (1996). Daidzein and genistein concentrations in human milk after soy consumption. Clin. Chem., 42, 955–964.
3. Gamache, P. H., and Acworth, I. N. (1998). Analysis of phytoestrogens and polyphenols in plasma, tissue, and urine using HPLC with coulometric detection. Proc. Soc. Exp. Biol. Med., 217, 274–280.
4. Gamache, P. H., Freeto, S. M. and Acworth, I. N. (1999). HPLC-electrochemical detection for phytoestrogen analysis: Isoflavones and lignans in human tissue and animal tissue. J. Med. Food, 2, 125–129.
5. Jones, D. J. L., Lim, C. K. Ferry, D. R., and Gescher, A. (1998). Determination of quercetin in human plasma by HPLC with spectrophotometric or electrochemical detection. Biomed. Chromatogr., 12, 232–235.
6. Lee, M-J., Wang, Z-Y., Li, H., Chen, L., Sun, Y., Gobbo, S., Balentine, D. A., and Yang, C. S. (1995). Analysis of plasma and urinary tea polyphenols in human subjects. Cancer Epidemiol. Bio. Prev., 4, 393–399.
7. Manach, C., Texier, O., Morand, C., Crespy, V. Regerat, F., Demigne, C., and Remesy, C. (1999). Comparison of the bioavailability of quercetin and catechin in rats. Free Rad. Biol. Med., 27, 1259–1266.
8. Nurmi, T., and Adlercreutz, H. (1999). Sensitive high-performance liquid chromatographic method for profiling phytoestrogens using coulometric electrode array detection: application to plasma analysis. Anal. Biochem., 274, 110–117.
9. Pan, Y., Anthony, M., and Clarkson, T. B. (1999). Effect of estradiol and soy phytoestrogens on choline acetyltransferase and nerve growth factor mRNAs in the frontal cortex and hippocampus of female rats. Proc. Soc. Exp. Biol. Med., 221, 118–125.
10. Acworth, I. N., and Gamache, P. H. (1996). The coulometric electrode array for use in HPLC analysis. Part 1: Theory. Am. Lab., 28, 33–38.
11. DeVries, J. H. M., Hollman, P. C. H., Meyboom, S., Buysman, M. N., Zock, P. L., van Staveren, W. A., and Katan, M. B. (1998). Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. Am. J. Clin. Nutr., 68, 60–65.

12. Manach, C., Texier, O., Regerat, F., Agullo, G., Demigne, C., and Remesy, C. (1996). Nutr. Biochem., 7, 375–380.
13. Goldberg, D. M., Tsang, E., Karumanchiri, A., Diamandis, E. P., Soleas, G., and Ng, E. (1996). Method to assay the concentrations of phenolic constituents of biological interest in wines. Anal. Chem., 68, 1688–1694.
14. Pezet, R., Pont, V., and Cuenat, P. (1994). J. Chromatogr. A, 663, 191–197.
15. Blache, D., Rustan I., Durand, P. Lesgards, G., and Loreau, N. (1997). Gas chromatographic analysis of resveratrol in plasma, lipoproteins and cells after in vitro incubations. J. Chromatogr. B, 702, 103–110.
16. Juan, M. E., Lamuela-Raventos, R. M., de la Torre-Boronat, M. C., and Planas, J. M. (1999). Determination of trans-resveratrol in plasma by HPLC. Anal. Chem., 71, 747–750.
17. Whitehead, T. P., Robinson, D. Allaway, S., Syms, J., and Hale, A. (1995). Effect of red wine ingestion on the antioxidant capacity of serum [see comments] Clin. Chem., 41, 32–35.
18. Zhu, Z., Klironomos, G., Vachereau, A., Neirinck, L., and Goodman, D. W. (1999). J. Chromatogr. B, 724, 389–392.
19. Manach, C., Morand, C., Texier, O., Favier, M-L., Agullo, G., Demigne, C. Regerat, F., and Remesy, C. (1995). Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. J. Nutr., 125, 1911–1922.

Ordering Information

In the U.S., call (800) 346-6390 or contact the Thermo Fisher Scientific Regional Office nearest you. Outside the U.S., order through your local Thermo Fisher Scientific office or distributor. Refer to the following part numbers.

Product Description	Part Number
HPG-3400RS Biocompatible Binary Rapid Separation Pump with Two Solvent Selector Valves	5040.0046
WPS-3000TBRS Biocompatible Rapid Separation	5841.0020
CoulArray, Model 5600A – 16 Channel	70-4334
CoulArray Organizer with Temp. Control	70-4340T
Accessory Kit, CoulArray Detector to Thermo Scientific™ Dionex™ UltiMate™ 3000 System	70-9191

www.thermofisher.com/chromatography

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Brazil +55 11 3731 5140
Canada +1 800 530 8447
China 800 810 5118 (free call domestic) 400 650 5118
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 9 3291 0200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591
Japan +81 6 6885 1213
Korea +82 2 3420 8600
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00
Russia/CIS +43 1 333 50 34 0
Singapore +65 6289 1190
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
Taiwan +886 2 8751 6655
UK/Ireland +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

AB71165-EN 0716S