**Technical detail for measurement of urinary metabolites of phytoestrogens**

In the present study, by using core-shell C6 phenyl chromatography, a column switching approach was modified from an established LC-MS/MS method(1) without the use of extraction or the pre-concentration techniques inherent in many existing methods.

Briefly, 40 μL urine sample were mixed with 20 μL of an enzyme solution freshly prepared by dissolving 4 mg of β-glucuronidase (Helix pomatia, Sigma) in 2 mL of 0.3M sodium acetate buffer (pH 5.0). This was incubated at 40 °C with shaking for 3 hours. After 10 minutes of cooling down, it was mixed with 20 μL of internal standard mixture (indole-3-acetamide and 4-hydroxybenzophenone, each 10 μg/mL) and 40 μL of methanol. After centrifugation, 20 μL of the filtrate was introduced onto a pre-column (Gemini C6 phenyl, 3 µm, 30 x 2 mm i.d.) and gradient separation was performed on an analytical column (core-shell C6–phenyl, 2.6 µm, 150 x 4.6mm i.d., Phenomenex, USA). By applying the column swapping technique, we enabled the simultaneous analysis of one sample on the above column while a second, identical column is flushed and equilibrated within 20 min. The key component of the mobile phase was 0.15% (v/v) formic acid and the two organic modifiers added were acetonitrile and methanol with concentration initiated at 10% and 0%, raised to 17% and 21% at 9 min, to 19% and 24% at 14 min and hold for the next 6 min. The quantification limits (N/S=5) for enterolactone, enterodiol, and equol using fluorimetric mode (ex. λ280nm, em. λ310 nm) were 10, 2, and 1 µg/L; respectively. Urinary concentrations of daidzein, glycitein and genistein above 20 µg/L could be determined using photodiode array detection at UV λ248, 256 and 260 nm; respectively.

Reference

1. Parker DL, Rybak ME, Pfeiffer CM (2012) Phytoestrogen biomonitoring: an extractionless LC-MS/MS method for measuring urinary isoflavones and lignans by use of atmospheric pressure photoionization (APPI). Anal Bioanal Chem **402**, 1123-1136.

**Supplemental Table 1.** Odds ratio (95% confidence interval) for incident type 2 diabetes according to total urine isoflavones and lignans metabolites stratified by sex

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Quartiles** | | | | **P for trenda** |
| **Q1** | **Q2** | **Q3** | **Q4** |
| **Isoflavones** |  |  |  |  |  |
| **Men** |  |  |  |  |  |
| Median (IQR)b | 0.82 (0.56-1.11) | 2.33 (1.82-3.12) | 5.80 (4.55-8.16) | 18.9 (14.1-28.0) |  |
| Cases/Control | 70/66 | 59/59 | 51/59 | 55/51 |  |
| Multivariate Model 1c | 1.00 | 0.94 (0.56-1.59) | 0.79 (0.47-1.33) | 1.00 (0.59-1.69) | 0.88 |
| Multivariate Model 2d | 1.00 | 0.85 (0.47-1.55) | 0.76 (0.42-1.37) | 0.87 (0.48-1.57) | 0.83 |
| Multivariate Model 3e | 1.00 | 0.84 (0.46-1.55) | 0.76 (0.41-1.38) | 0.76 (0.41-1.41) | 0.50 |
| **Women** |  |  |  |  |  |
| Median (IQR)b | 0.86 (0.68-1.18) | 2.12 (1.80-2.82) | 6.27 (4.88-8.09) | 20.1 (13.9-33.4) |  |
| Cases/Control | 92/75 | 73/82 | 79/82 | 85/90 |  |
| Multivariate Model 1c | 1.00 | 0.74 (0.48-1.14) | 0.77 (0.50-1.20) | 0.77 (0.50-1.19) | 0.58 |
| Multivariate Model 2d | 1.00 | 0.70 (0.43-1.15) | 0.74 (0.45-1.22) | 0.76 (0.46-1.24) | 0.71 |
| Multivariate Model 3e | 1.00 | 0.71 (0.43-1.17) | 0.74 (0.45-1.23) | 0.76 (0.46-1.25) | 0.67 |
| **Lignans** |  |  |  |  |  |
| **Men** |  |  |  |  |  |
| Median (IQR)b | 0.16 (0.10-0.26) | 1.02 (0.76-1.34) | 2.74 (2.18-3.52) | 7.11 (5.26-9.75) |  |
| Cases/Control | 63/62 | 60/74 | 67/51 | 45/48 |  |
| Multivariate Model 1c | 1.00 | 0.77 (0.47-1.27) | 1.26 (0.78-2.02) | 0.87 (0.50-1.53) | 0.93 |
| Multivariate Model 2d | 1.00 | 0.85 (0.48-1.49) | 1.31 (0.76-2.26) | 0.97 (0.51-1.85) | 0.73 |
| Multivariate Model 3e | 1.00 | 0.83 (0.47-1.46) | 1.36 (0.78-2.33) | 0.89 (0.46-1.74) | 0.88 |
| **Women** |  |  |  |  |  |
| Median (IQR)b | 0.13 (0.10-0.27) | 1.00 (0.73-1.33) | 2.71 (2.22-3.41) | 7.57 (5.48-11.0) |  |
| Cases/Control | 96/79 | 64/67 | 74/90 | 95/93 |  |
| Multivariate Model 1c | 1.00 | 0.80 (0.50-1.26) | 0.73 (0.48-1.10) | 0.84 (0.54-1.30) | 0.65 |
| Multivariate Model 2d | 1.00 | 0.96 (0.57-1.59) | 0.99 (0.62-1.57) | 0.98 (0.60-1.61) | 0.99 |
| Multivariate Model 3e | 1.00 | 0.95 (0.57-1.59) | 0.99 (0.62-1.58) | 0.96 (0.58-1.57) | 0.91 |

a Linear trend was tested by treating the median of quartiles as a continuous variable;

b IQR: interquartile range. The values are expressed as nmol/mg creatinine;

cMultivariate model 1: adjusted for age at biospecimen collection (continuous) and fasting duration (≤6 and >6 hours);

dMultivariate model 2: further adjusted for education level, body mass index, physical activity, smoking, alcohol use, hypertension history, and total energy intake;

eMultivariate model 3: further adjusted for vegetable intake, fruits and related juices, saturated fatty acid, and omega-3 fatty acid.

**Supplemental Table 2.** Odds ratio (95% confidence interval) for incident type 2 diabetes according to individual urine metabolites of isoflavones and lignans

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Quartiles** | | | | **P for trenda** |
| **Q1** | **Q2** | **Q3** | **Q4** |
| **Daidzein** |  |  |  |  |  |
| Median (IQR)b | 0.26 (0.17-0.31) | 0.73 (0.58-0.95) | 2.45 (1.72-3.09) | 9.27 (6.44-15.8) |  |
| Cases/Control | 167/141 | 110/141 | 147/141 | 140/141 |  |
| Multivariate Model 1c | 1.00 | 0.66 (0.47-0.92) | 0.86 (0.63-1.20) | 0.82 (0.58-1.15) | 0.88 |
| Multivariate Model 2d | 1.00 | 0.68 (0.46-0.99) | 0.85 (0.59-1.23) | 0.79 (0.54-1.16) | 0.72 |
| Multivariate Model 3e | 1.00 | 0.67 (0.45-0.98) | 0.82 (0.57-1.19) | 0.75 (0.51-1.10) | 0.52 |
| **Genistein** |  |  |  |  |  |
| Median (IQR)b | 0.31 (0.19-0.36) | 0.74 (0.61-0.90) | 1.81 (1.45-2.20) | 5.30 (3.79-8.90) |  |
| Cases/Control | 161/141 | 156/141 | 116/141 | 131/141 |  |
| Multivariate Model 1c | 1.00 | 0.96 (0.69-1.34) | 0.68 (0.48-0.97) | 0.80 (0.57-1.12) | 0.19 |
| Multivariate Model 2d | 1.00 | 0.87 (0.60-1.27) | 0.58 (0.39-0.87) | 0.81 (0.55-1.19) | 0.42 |
| Multivariate Model 3e | 1.00 | 0.87 (0.60-1.26) | 0.57 (0.38-0.85) | 0.78 (0.53-1.15) | 0.32 |
| **Glycitein** |  |  |  |  |  |
| Median (IQR)b | 0.07 (0.05-0.08) | 0.16 (0.13-0.19) | 0.23 (0.23-0.32) | 1.04 (0.66-1.77) |  |
| Cases/Control | 157/141 | 123/141 | 144/141 | 140/141 |  |
| Multivariate Model 1c | 1.00 | 0.77 (0.55-1.09) | 0.90 (0.65-1.25) | 0.87 (0.63-1.22) | 0.80 |
| Multivariate Model 2d | 1.00 | 0.72 (0.48-1.06) | 0.99 (0.69-1.44) | 0.88 (0.61-1.29) | 0.93 |
| Multivariate Model 3e | 1.00 | 0.74 (0.50-1.09) | 0.98 (0.68-1.42) | 0.85 (0.58-1.24) | 0.69 |
| **Equol** |  |  |  |  |  |
| Median (IQR)b | 0 (0-0) | 0.01 (0.01-0.01) | 0.15 (0.06-0.45) | 3.60 (2.02-8.30) |  |
| Cases/Control | 161/141 | 128/141 | 142/141 | 133/141 |  |
| Multivariate Model 1c | 1.00 | 0.79 (0.57-1.09) | 0.89 (0.64-1.23) | 0.82 (0.59-1.14) | 0.57 |
| Multivariate Model 2d | 1.00 | 0.85 (0.59-1.23) | 0.98 (0.68-1.42) | 0.95 (0.66-1.38) | 0.92 |
| Multivariate Model 3e | 1.00 | 0.87 (0.60-1.26) | 0.97 (0.67-1.41) | 0.97 (0.67-1.41) | 0.88 |
| **Enterodiol** |  |  |  |  |  |
| Median (IQR)b | 0.02 (0.01-0.03) | 0.13 (0.10-0.18) | 0.36 (0.28-0.46) | 0.99 (0.70-1.79) |  |
| Cases/Control | 138/141 | 143/141 | 149/141 | 134/141 |  |
| Multivariate Model 1c | 1.00 | 1.04 (0.75-1.45) | 1.07 (0.77-1.49) | 0.98 (0.70-1.36) | 0.77 |
| Multivariate Model 2d | 1.00 | 1.35 (0.93-1.97) | 1.27 (0.87-1.84) | 1.18 (0.81-1.72) | 0.88 |
| Multivariate Model 3e | 1.00 | 1.32 (0.90-1.93) | 1.23 (0.84-1.81) | 1.12 (0.76-1.64) | 0.90 |
| **Enterolactone** |  |  |  |  |  |
| Median (IQR)b | 0.09 (0.05-0.11) | 0.68 (0.43-0.92) | 2.24 (1.73-2.79) | 6.35 (4.62-9.23) |  |
| Cases/Control | 152/141 | 134/141 | 141/141 | 137/141 |  |
| Multivariate Model 1c | 1.00 | 0.90 (0.65-1.23) | 0.96 (0.70-1.31) | 0.90 (0.64-1.26) | 0.68 |
| Multivariate Model 2d | 1.00 | 0.99 (0.69-1.42) | 0.99 (0.70-1.41) | 1.02 (0.70-1.49) | 0.87 |
| Multivariate Model 3e | 1.00 | 0.99 (0.69-1.42) | 1.02 (0.72-1.45) | 1.00 (0.68-1.47) | 0.98 |

a Linear trend was tested by treating the median of quartiles as a continuous variable;

b IQR: interquartile range. The values are expressed as nmol/mg creatinine;

cMultivariate model 1: adjusted for age at biospecimen collection (continuous) and fasting duration (≤6 and >6 hours);

dMultivariate model 2: further adjusted for education level, body mass index, physical activity, smoking, alcohol use, hypertension history, and total energy intake;

eMultivariate model 3: further adjusted for vegetable intake, fruits and related juices, saturated fatty acid, and omega-3 fatty acid.

**Supplemental Figure 1.** Study profile.

1st Follow-up

2nd Follow-up

SCHS Baseline

Full interview:

- Diet by FFQ

- T2D status, hypertension status, etc.

Biospecimen collection

Laboratory measurements:

- Urinary **phytoestrogens**

- HbA1C, lipids, CRP, etc.

Update interview:

- T2D and hypertension status, smoking, drinking, exercise, menstrual history, etc.

Update interview:

- **T2D status**, etc.

Total SCHS population

Excluded:

* T2D
* CHD, stroke, cancer

Excluded:

* T2D
* No urine sample
* CHD, stroke, cancer

New T2D

No T2D

Excluded:

* CHD, stroke, cancer before T2D

Excluded:

* HbA1c ≥ 6.0%
* Unmatched

Cases

Controls

**Study Population**

**Data collection**

**Stages**

**Abbreviations**

BMI: body mass index; BP: blood pressure; FFQ: food frequency questionnaire; SCHS: Singapore Chinese Health Study; T2D: type 2 diabetes.

**Supplemental Figure 2.** Dose-response relationship between isoflavone metabolites and risk of type 2 diabetes.

A: all study case-control pairs;B: subset of cases with HbA1c < 6.5% and matched controls; C: subset of cases with HbA1c ≥ 6.5% and matched controls. Multivariate conditional logistic regression models were used with adjustment for age at biospecimen collection (continuous) and fasting duration (≤ 6 and > 6 hours), education level, body mass index, physical activity, smoking, alcohol use, hypertension history, and total energy intake, vegetable intake, fruits and related juices, saturated fatty acid, and omega-3 fatty acid. Solid red lines are ORs and dashed lines are 95% CI.

|  |  |
| --- | --- |
| **A** | **RCS 2.tif** |
| **B** | **RCS_HbA1c_Low 2.tif** |
| **C** | **RCS_HbA1c_High 2.tif** |