Technical detail for measurement of urinary metabolites of phytoestrogens

In the present study, by using core-shell C_6 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 C_6 phenyl, 3 μm, 30 x 2 mm i.d.) and gradient separation was performed on an analytical column (core-shell C_6–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 trend[a]
	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 1[c]	1.00	0.94 (0.56-1.59)	0.79 (0.47-1.33)	1.00 (0.59-1.69)	0.88
Multivariate Model 2[d]	1.00	0.85 (0.47-1.55)	0.76 (0.42-1.37)	0.87 (0.48-1.57)	0.83
Multivariate Model 3[e]	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 1[c]	1.00	0.74 (0.48-1.14)	0.77 (0.50-1.20)	0.77 (0.50-1.19)	0.58
Multivariate Model 2[d]	1.00	0.70 (0.43-1.15)	0.74 (0.45-1.22)	0.76 (0.46-1.24)	0.71
Multivariate Model 3[e]	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 1[c]	1.00	0.77 (0.47-1.27)	1.26 (0.78-2.02)	0.87 (0.50-1.53)	0.93
Multivariate Model 2[d]	1.00	0.85 (0.48-1.49)	1.31 (0.76-2.26)	0.97 (0.51-1.85)	0.73
Multivariate Model 3[e]	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 1[c]	1.00	0.80 (0.50-1.26)	0.73 (0.48-1.10)	0.84 (0.54-1.30)	0.65
Multivariate Model 2[d]	1.00	0.96 (0.57-1.59)	0.99 (0.62-1.57)	0.98 (0.60-1.61)	0.99
Multivariate Model 3[e]	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;

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

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

[e] Multivariate 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 trend[a] |
	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 1[c]	1.00	0.66 (0.47-0.92)	0.86 (0.63-1.20)	0.82 (0.58-1.15)	0.88
Multivariate Model 2[d]	1.00	0.68 (0.46-0.99)	0.85 (0.59-1.23)	0.79 (0.54-1.16)	0.72
Multivariate Model 3[e]	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 1[c]	1.00	0.96 (0.69-1.34)	0.68 (0.48-0.97)	0.80 (0.57-1.12)	0.19
Multivariate Model 2[d]	1.00	0.87 (0.60-1.27)	0.58 (0.39-0.87)	0.81 (0.55-1.19)	0.42
Multivariate Model 3[e]	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 1[c]	1.00	0.77 (0.55-1.09)	0.90 (0.65-1.25)	0.87 (0.63-1.22)	0.80
Multivariate Model 2[d]	1.00	0.72 (0.48-1.06)	0.99 (0.69-1.44)	0.88 (0.61-1.29)	0.93
Multivariate Model 3[e]	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 1[c]	1.00	0.79 (0.57-1.09)	0.89 (0.64-1.23)	0.82 (0.59-1.14)	0.57
Multivariate Model 2[d]	1.00	0.85 (0.59-1.23)	0.98 (0.68-1.42)	0.95 (0.66-1.38)	0.92
Multivariate Model 3[e]	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 1[c]	1.00	1.04 (0.75-1.45)	1.07 (0.77-1.49)	0.98 (0.70-1.36)	0.77
Multivariate Model 2[d]	1.00	1.35 (0.93-1.97)	1.27 (0.87-1.84)	1.18 (0.81-1.72)	0.88
Multivariate Model 3[e]	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 1[c]	1.00	0.90 (0.65-1.23)	0.96 (0.70-1.31)	0.90 (0.64-1.26)	0.68
Multivariate Model 2[d]	1.00	0.99 (0.69-1.42)	0.99 (0.70-1.41)	1.02 (0.70-1.49)	0.87
Multivariate Model 3[e]	1.00	0.99 (0.69-1.42)	1.02 (0.72-1.45)	1.00 (0.68-1.47)	0.98

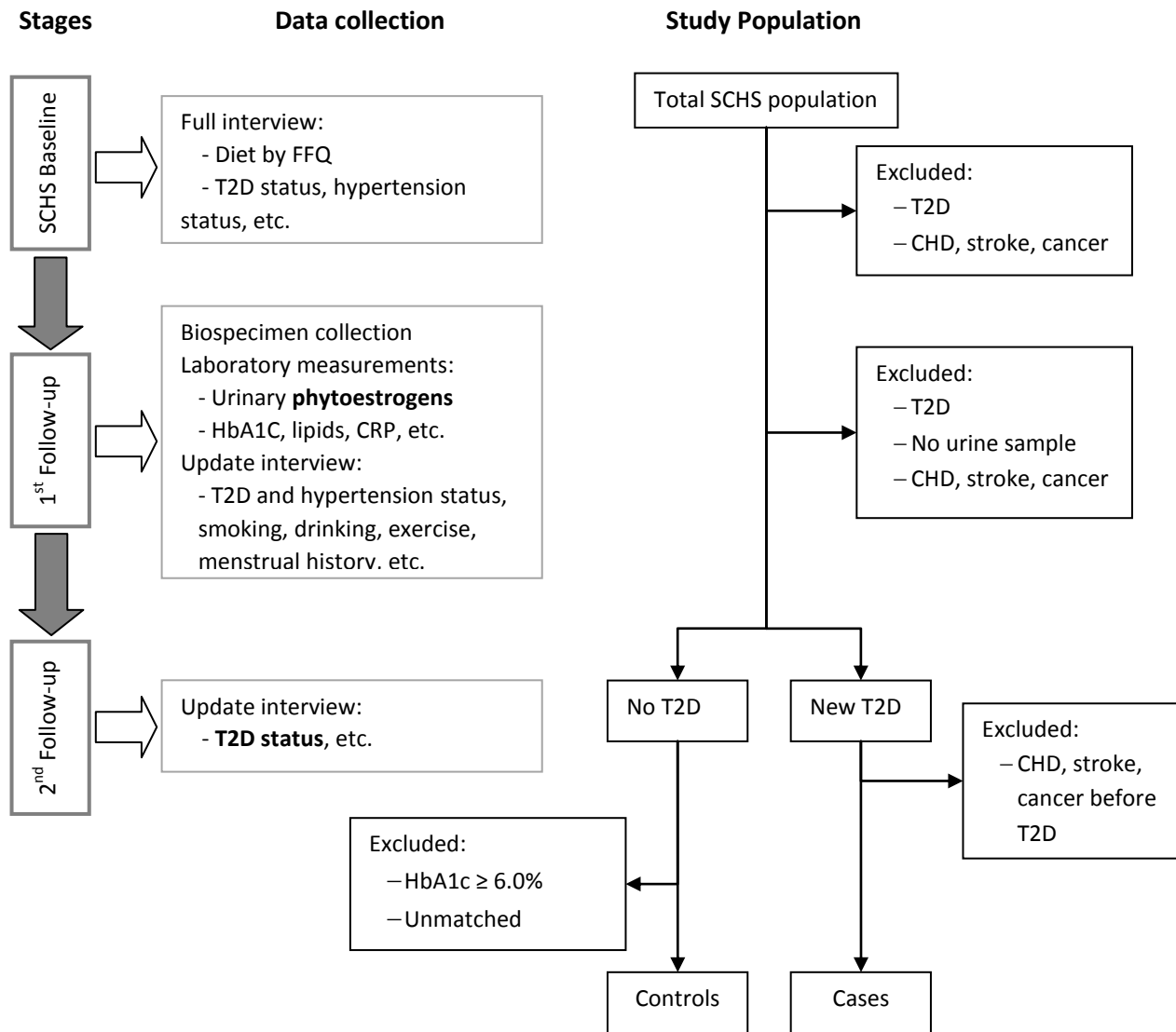[a] Linear trend was tested by treating the median of quartiles as a continuous variable;

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

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

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

[e] Multivariate model 3: further adjusted for vegetable intake, fruits and related juices, saturated fatty acid, and omega-3 fatty acid.

Supplemental Figure 1. Study profile.

Stages	Data collection	Study Population

Stages

SCHS Baseline

1st Follow-up

2nd Follow-up

Data collection

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.

Study Population

Total SCHS population

Excluded:
 − T2D
 − CHD, stroke, cancer

Excluded:
 − T2D
 − No urine sample
 − CHD, stroke, cancer

No T2D

New T2D

Excluded:
 − CHD, stroke, cancer before T2D

Excluded:
 − HbA1c ≥ 6.0%
 − Unmatched

Controls

Cases

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.