Research Article

Soy Isoflavones in Epidemiological Serum Samples: What are the Optimal Time Window and Concentration Cutoffs for Assignment of Equol Producer Status?

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Abstract

Background: The ability to produce equol from daidzein may explain observed inconsistencies in isoflavones' health effects. Reliable identification of Equol Producers (EqPs) in non-clinical studies is limited by poor understanding of pharmacokinetics in producers and background concentrations in Equol Non-producers (EqNPs).

Objective: To characterize equol pharmacokinetics and identify optimal time windows and concentration cutoffs for assignment of EqP phenotype.

Design: An optimal time window was identified using a pharmacokinetic study (N=10) with sampling every 2-3hrs for 24hrs following ingestion of 4mg daidzein. An optimal cut off was conducted through analysis of samples collected at baseline, 3 stages following 1 month intake of: (1) 38.5mg/day, (2) 77.0mg/day; (3) 115.4mg/day daidzein aglycone equivalents; and post one-month washout period (N=7).

Result: Serum equol peaked between 12-24 hrs following isoflavone intake. A 22nM threshold clearly distinguished between samples collected from EqPs and EqNPs 12-24 hours post-ingestion, while ratios of Equol/Daidzein did not. Following high isoflavone intake, no dose response in equol concentrations was observed in EqNPs.

Conclusion: Serum equol concentrations greater than 22nM reliably indicate intestinal microflora ability to metabolize daidzein to Equol (EqP phenotype). Sampling 12-24hr post-ingestion maximizes equol producer status assignment accuracy.

Keywords: Equol, Isoflavones, Soy, Serum, Pharmacokinetics

Abbreviations

D: Daidzein; G: Genistein; Eq: Equol; EqP: Equol Producer; EqNP: Equol Non-producer.

Introduction

Research suggests that many health benefits of isoflavones may depend on an individual's ability to metabolize the isoflavone Daidzein (D) into the isoflavan Equol (Eq), because of Eq's greater estrogenic activity and affinity for both estrogen receptors [1,2]. The ability to metabolize Eq from D depends on the presence of certain intestinal microflora [3,4]. People who harbor the necessary microflora to metabolize daidzein into equol are called Equol Producers (EqP) and show measurable concentrations of equol in blood and urine following ingestion of soy or other daidzein-containing foods or supplements, and those who do not are considered Equol Non-Producers (EqNP). The prevalence of equol producers varies from 30% to 59% in human populations, and is higher in Asian and vegetarian populations [5-9].

No published data exist on the average or maximum background serum concentrations of equol in non-producers, or on the pharmacokinetics of equol that would enable optimal sampling in the post-ingestion window when equol peaks above background concentrations in producers. Thus, reliable guidelines for assigning equol producer status based on serum samples, the most routinely collected samples in epidemiological studies, are nonexistent.

Methods for assessing EqP ability generally involve analysis of fecal microflora [3] or a soy or daidzein challenge test followed by 24-hour urine analysis [8]. Although some researchers have found strong correlations between urinary Eq excretion and plasma Eq concentrations [10], others argue that while urinary isoflavone concentrations may reflect average exposure, they correlate poorly with maximal serum concentrations [5], and thus may be of limited utility for assessing systemic bioavailability and cellular and organ exposure. Furthermore, collection of fecal or 24-hour urine samples is rarely feasible in large-scale epidemiological studies examining the effects of soy on cholesterol, heart disease, menopausal symptoms, and osteoporosis. Thus it is important to develop algorithms for reliable assignment of EqP status from serum samples.

Pharmacokinetic studies have observed peaks in blood Genistein (G) and daidzein concentrations in the first 8 hours post-ingestion and reported maximal Eq concentrations at 24 hours post-ingestion [11-13]. However, the report of an Eq maximum at 24 hrs may be an artifact of sampling frequency and schedule. Most studies sampled

frequently during the first 12 hours, but subsequently only every 12 hours, presumably because of researchers' and participants' need for sleep. Thus the observed Eq peak at 24 hours could mean that Eq reached maximum concentration anywhere between 12-36 hours. The use of our validated finger-prick dried blood spot method [14] represents a significant advance for pharmacokinetic studies of isoflavones and metabolites, as self-sampling by participants at home enables more frequent collection throughout a 24-hour period.

To identify the optimal time window for assigning EqP status from serum samples collected in epidemiological studies, we carried out pharmacokinetic studies with sampling every 2-3 hours for the 24 hours following isoflavone ingestion. Since background levels of equol may be observed in EqNP due to the presence of equol in cow's milk (metabolized by cows from daidzein in forage legumes), it is important to establish a cutoff for equol in serum above which a person can be reliably considered to be an equol producer. To identify the optimal Eq serum concentration cutoff to reliably assign EqP status (i.e., to distinguish between EqNPs and EqPs), we conducted a study of repeated high dose isoflavone intake. This simulates a long-term daidzein challenge test and permits identification of maximal Eq concentration in EqNPs

Materials and Methods

Two formulations of purified isoflavones derived from soy hypocotyls were provided by Fuji Oil Company Ltd. (Osaka, Japan), containing a total of 2.08mg and 27.75mg isoflavones per 300mg tablet in a base of lactose (78.5%) and glycerin fatty acid esters (3.0%) in Formulations 1 and 2 respectively (Table 1). The study tablets used here had higher ratios of D:G (approximately 4.8:1 and 2.8:1) than the 1:1.5 ratio typically observed in foods [15] in order to administer maximum D substrate for assessing equol production ability. Study protocols were approved by the Ethical Committee of the Life Science Foundation, and Tokyo University of Agriculture. Informed consent was obtained from all study participants and the procedures followed were in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Optimal time window study

Initially, a detailed pharmacokinetic case study using Formulation 1 tablets was performed to establish feasibility and identify optimal sampling windows for Eq (data not shown). Subsequently, ten volunteers (6 females and 4 males, ages 23-61yrs) collected finger-prick dried blood spot samples at baseline, and then every 2-3 hours for a 24-hour period after consumption of 20.76mg isoflavones (10 Formulation 1 tablets) containing 4mg D, approximating the D intake from an epidemiologically-normal average meal among Japanese living in Japan [6,15-17]. Sample collection during the sleep period did not always conform to the every 2-hour sample protocol. The subjects ingested isoflavones before sleeping, to ensure reliable collection of samples during the 12-24 hour post-ingestion window (when Eq peaks). Participants were Japanese (3 females), Asian-American (1 male) and Caucasian-American (3 females and 3 males) and ranged in age from 23-62 years. Participants did not consume any soy products for the 24 hours prior to, or during, the protocol, and reported no antibiotic use in the previous 6 months. Blood spot samples were collected as described previously [14], allowed to dry for at least 4 hours at room temperature, then stored in participants' refrigerators until they could be delivered to the laboratory, where they were stored at –20°C until analysis.

Optimal concentration cutoff study

Fasting serum and urine samples were collected from 7 college-aged (21-22 years) Japanese women in the morning and measured for D, G, and Eq at baseline, after 1 month of 111mg/day isoflavone intake (4 Formulation 2 tablets consumed at breakfast), a second month of 222mg/day isoflavone (4 tablets at breakfast and at dinner), a third month of 333mg/day isoflavone (4 tablets at breakfast, lunch and dinner), and after a final month of washout (no isoflavone tablets). Dietary isoflavone intake was limited to 20mg/day throughout the study.

Analytic and Statistical Methods

All serum and blood spot samples in these studies were measured by our HPLC-CEAD method [14]. Intra-assay CVs ranged from 3.3%-7.2% for 3 pooled controls with Eq concentrations of 69.4nM, 562.2nM and 1189nM. Inter-assay CVs ranged from 13.2-20.2% for the same controls. Each sample analytic run used a 6-point standard curve at the beginning of each run, and standards, 3 controls (low, medium and high), and blanks at the end of the run. Samples from the detailed pharmacokinetic study were run in a single batch. Samples from the 10 participants in the 'Optimal Time Window Study' were run in 4 batches. Samples from the 7 participants in the optimal concentration cutoff study were run in 2 batches. Samples with non-detectable Eq concentrations were assigned the detection limit value of 0.37nM (defined as the concentration at which the signal/noise ratio equals 3:1 on the dominant channel). The $\log_{10}$ ratio of Eq to D was calculated for all pharmacokinetic samples, and group differences between variables were examined using the non-parametric Kruskal-Wallis and Mann-Whitney tests. P-values (2-tailed) of less than 0.05 were considered significant. Results were plotted in rank order and

Table 1 Composition of isoflavones in study tablet formulations (per 300mg tablet)[a]

Isoflavone	Formulation 1 mg (Aglycone Equivalent)		Formulation 2 mg (Aglycone Equivalent)	
daidzin	0.46	(0.28)	9.35	(5.71)
genistin	0.23	(0.14)	1.71	(1.07)
glycitin	0.76	(0.48)	4.43	(2.82)
malonyl daidzin	0.01	(0.01)	7.11	(3.60)
malonyl genistin	0.01	(0.00)	1.70	(0.89)
malonyl glycitin	0.02	(0.01)	2.60	(1.39)
acetyl daidzin	0.19	(0.10)	0.51	(0.28)
acetyl genistin	0.00	(0.00)	0.13	(0.07)
acetyl glycitin	0.29	(0.17)	0.15	(0.09)
daidzein	0.01	(0.01)	0.03	(0.03)
genistein	0.00	(0.00)	0.01	(0.01)
glycitein	0.11	(0.11)	0.02	(0.02)
Total IFs	2.08	(1.31)	27.75	(15.98)
D to G ratio	2.81	(2.70)	4.79	(4.72)
One-time intake (# tablets)	10 tablets		4 tablets	
Total IFs/intake	20.76	(13.14)	111.00	(63.90)

[a]Composition analyzed by Fuji Oil Company Ltd (Osaka, Japan)

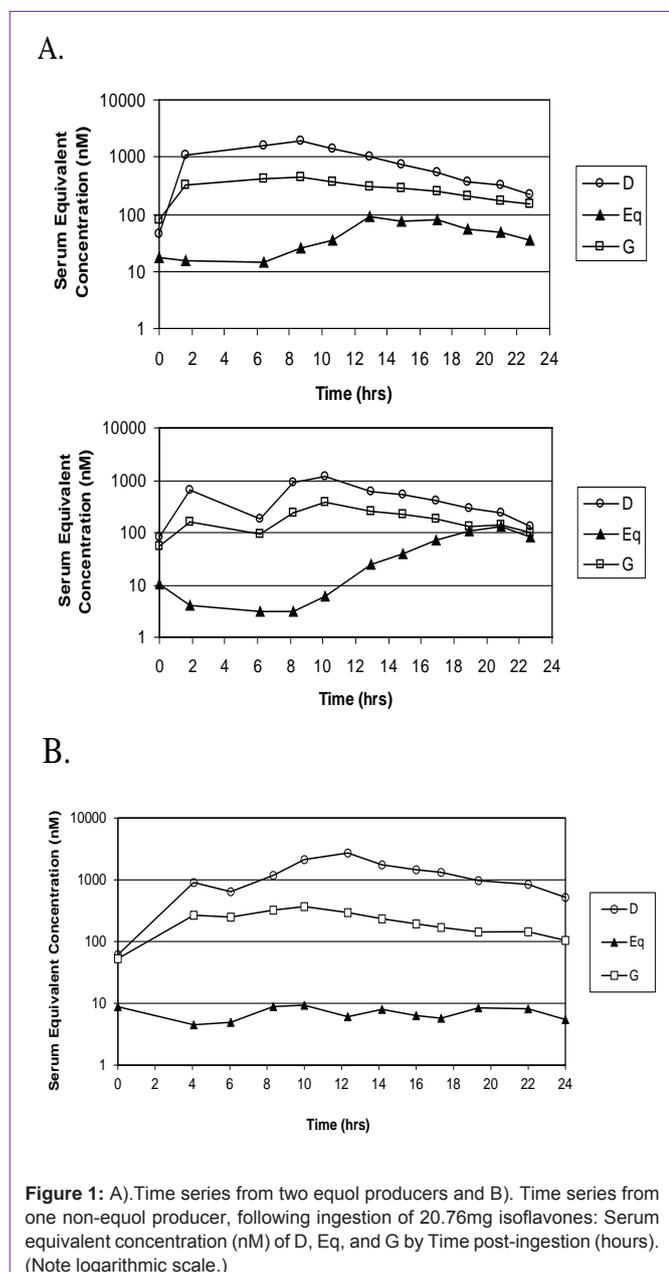examined to identify thresholds for equol producer status [8]. SPSS was used for analyses.

Results

Optimal time window study

Figure 1 shows representative time series for two EqPs and one EqNP. Table 2 contains participants' gender and age and ethnicity by EqP status. Table 3 contains descriptive statistics for D, G, Eq and $\log_{10}$ (Eq/D) for EqPs and EqNPs by time period (0-12 and 12-24 hours post-ingestion). In EqNPs, the highest Eq concentration achieved during the entire 24-hour period was 18.5nM. Only 3 of 10 participants (EqPs) had samples with high Eq concentrations, with most of these occurring 12-24 hours post-ingestion (Figure 2A). During this period, the lowest observed Eq concentration was 24.7nM in EqPs. Thus the midpoint value between highest observed Eq

Table 2: Participants' gender and age and ethnicity by EqP status.

EqP status	Gender	Age	Ethnicity
Y	F	27	Japanese
Y	F	51	Caucasian-American
Y	M	26	Caucasian-American
N	F	22	Caucasian-American
N	F	23	Caucasian-American
N	F	31	Japanese
N	F	61	Caucasian-American
N	M	28	Caucasian-American
N	M	45	Korean-American
N	F	58	Japanese

Y: Yes; N: No; M: Male; F: Female

concentration in EqNPs and lowest Eq concentration in EqPs in the 12-24 hr window was 22nM. Eq concentrations differed significantly between groups ($P = 6.4 \times 10^{-9}$), but D and G concentrations did not. While $\log_{10}$ (Eq/D) values also differed significantly between groups, significance was lower than for Eq concentration alone. Plots of Eq in rank order by group showed that the midpoint threshold of 22nM provided a clear upper limit for EqNP samples (Figure 2A), and all EqP samples in the 12-24 hour window were above this threshold. In contrast, plots of $\log_{10}$ (Eq/D) showed great overlap between groups (Figure 2B).

Optimal concentration cutoff study

Table 4 shows descriptive statistics for D, Eq, and G by isoflavone intake. Results from urine samples showed similar patterns (data not shown). Increases in D and G were observed in all participants indicating compliance with the protocol, although not increase following 1month of 333mg isoflavone intake compared to 222mg for 4 of 7 participants, suggesting that the limit of absorption may have been reached. Four out of 7 women showed dramatic increases in serum Eq after daily isoflavone intake, and were considered to be EqPs. Three women showed no increases in Eq and were considered to be EqNPs. Only one EqNP had detectable concentrations at all time points, starting from a maximum of 29.5nM at baseline and decreasing throughout the study. The other two EqNPs had detectable Eq only after 333mg isoflavone consumption, but concentrations were less than 5nM.

Overall

In the two studies combined, 7 of 17 participants (41%) were EqPs and provided 101 samples, while 10 of 17 were EqNPs (59%) and provided 45 samples for analysis. Although these 101 samples were repeated measures on 17 individuals, they can be used heuristically to estimate false positive and negative rates of EqP assignment for single measurements using a particular threshold. Using an Eq threshold of 22nM, none of the 86 EqNP samples in the pharmacokinetic study and only 1 of 101 EqNP samples in the two studies combined would have been identified as coming from an EqP, giving a false positive rate of 1.0%. Using the same threshold, the false negative rate (assignment of a true EqP as an EqNP) would have been 0% (0 of 14 samples) for samples collected 12-24 hours post-ingestion in the pharmacokinetic study, or 3.3% (1 of 30 samples) when samples collected from EqPs 12-24 hours post-ingestion in the pharmacokinetic study were

Figure 1: A).Time series from two equol producers and B). Time series from one non-equol producer, following ingestion of 20.76mg isoflavones: Serum equivalent concentration (nM) of D, Eq, and G by Time post-ingestion (hours). (Note logarithmic scale.)

Table 3 Serum equivalent concentrations of daidzein, equol and genistein and $\log_{10}(Eq/D)$ in dried blood spots following ingestion of 20 mg IF supplement by equol producer status and time period, and P-values for group differences.
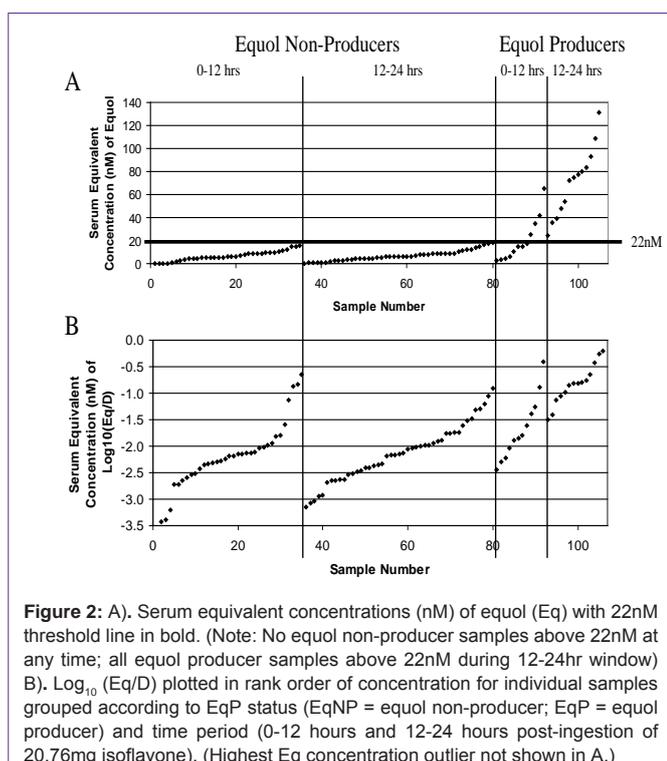
EqP Status	Sample Time Period Post-ingestion	N_s[a]	D (nM)			Eq (nM)			G (nM)			Log10(Eq/D)		
			Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max
Equol	0-12 hours	12	44.9	1049.0	1974.8	3.0	14.9	65.2	54.8	348.1	501.9	-2.4	-1.8	-0.4
Producers (N=3)	12-24 hours	14	135.0	468.5	2361.0	24.7	76.3	347.9	101.1	218.3	1015.1	-1.5	-0.8	-0.2
Equol Non-	0-12 hours	41	26.8	1012.4	4194.5	0.4	5.8	15.3	4.3	190.3	552.4	-0.6	-2.2	-0.7
Producers (N=7)	12-24 hours	45	136.9	755.6	3516.8	0.0	6.0	18.5	4.4	143.7	731.8	-3.1	-2.1	-0.9
Statistical differences (P-values) between groups by non-parametric Kruskal-Wallis test														
4 groups				0.176			6.415×10^{-9}			0.155			1.011×10^{-7}	
3 groups[b]				0.188			1.235×10^{-9}			0.082			4.299×10^{-8}	

[a]N_s is number of samples analyzed per group, defined by equol producer status and time period post-ingestion

[b]All Equol Non-Producer samples (0-12 hours and 12-24 hours post-ingestion groups) combined

Figure 2: A). Serum equivalent concentrations (nM) of equol (Eq) with 22nM threshold line in bold. (Note: No equol non-producer samples above 22nM at any time; all equol producer samples above 22nM during 12-24hr window) B). $\log_{10}$ (Eq/D) plotted in rank order of concentration for individual samples grouped according to EqP status (EqNP = equol non-producer; EqP = equol producer) and time period (0-12 hours and 12-24 hours post-ingestion of 20.76mg isoflavone). (Highest Eq concentration outlier not shown in A.)

combined with non-baseline samples from EqPs in the high dose isoflavone study.

Discussion

Guidelines for assessment of equol producer ability based on serum samples are greatly needed, as large-scale epidemiological studies often collect only singlicate blood samples. Our pharmacokinetic study suggests that using a threshold of 22nM serum Eq appears to minimize false positives, i.e. assignment of EqNP as EqP. With samples above this threshold, individuals are likely to harbor sufficient intestinal microflora to metabolize daidzein into equol in a dose-response fashion. The challenge is to minimize the rate of

false negatives (assignment of EqP as EqNP) due to pharmacokinetic constraints or insufficient substrate.

Most previous pharmacokinetic studies suggested that Eq peaks at 24 hours, but all were limited by collection of samples only at 12, 24, 36 and 48 hours [11,12,18] and thus likely missed the true peak occurring between 12-24 hours. To our knowledge, our study constitutes the first detailed investigation of Eq pharmacokinetics 12-24 hours post-ingestion, and suggests that Eq peaks around 16 hours. In a detailed case study following high isoflavone intake, a large Eq peak was observed at 16 hours, but small peaks were observed around 6 hours (data not shown), similar to the 6-10 hour peak reported in one study that did not sample between 12-24 hours [19]. Thus, if EqP status is of interest, sampling should occur 12-24 hours post-ingestion to maximize the probability of accurately assigning EqP status. All EqP samples in the 12-24 hour post-ingestion window were greater than 22nM.

Thus we propose the guidelines below for assigning equol producer status in observational and epidemiological studies when single serum samples are collected with matched 24-hour dietary records:

1. If [Eq] >22nM, then assign EqP status (with estimated 1% false positive rate)

2. If [Eq] <22nM, then examine dietary records:

a. If dietary isoflavone intake equivalent to at least 4.0mg D aglycone occurred 12-24 hours before sampling, then assign EqNP status (with estimated 0-3% false negative rate)

b. If dietary intake equivalent to less than 4.0mg D aglycone occurred 12-24 hours before sampling, then assign uncertain EqP status. Repeat measures on these individuals should be performed when possible.

Setchell and Cole proposed a 20nM serum threshold for distinguishing between EqPs and EqNPs based on data from 20 EqPs and 21 EqNPs [8], supporting the threshold of 22nM identified

Table 4 Range of serum concentrations in equol producers and non-producers by research stage (IF intake amount)

EqP Status	Research Stage IF Intake	Sample Size[a] N (N$_{Eq}$)	D (nM)			Eq (nM)[b]			G (nM)		
			Min	Med	Max	Min	Med	Max	Min	Med	Max
	Baseline	3	11.2	14.9	30.4	13.9	15.9	35.2	19.4	27.2	61.9
	111 mg/day	4	44.1	158.9	285.2	93.0	208.0	367.6	49.8	85.3	194.1
Equol Producers	222 mg/day	4	209.9	633.0	852.3	13.0	465.4	563.5	48.3	102.5	253.9
	333 mg/day	4	305.4	1138.0	1333.9	340.1	527.3	783.7	85.2	144.3	561.0
	Post 1mo	4 (3)	13.7	17.9	44.1	33.7	121.4	181.5	19.8	32.9	174.9
	Baseline	3 (1)	5.8	12.3	21.2	29.5	29.5	29.5	18.2	30.7	64.1
Equol Non-Producers	111 mg/day	3 (1)	50.5	214.2	362.8	20.3	20.3	20.3	70.3	97.7	168.4
	222 mg/day	3 (1)	639.1	657.5	842.7	9.0	9.0	9.0	82.7	212.8	219.0
	333 mg/day	3	327.7	532.2	845.0	2.5	4.0	4.3	100.7	103.9	310.2
	Post 1mo	3 (1)	13.3	32.0	38.5	6.5	6.5	6.5	18.2	74.3	142.3

[a] N = sample size out of 7 total participants (4 EqP and 3 EqNP). N$_{Eq}$ = Sample size for Eq analyses if less than N, due to undetectable concentrations

[b] Equol concentrations significantly different between EqP and EqNP (P<0.001 by Mann-Whitney nonparametric test for all samples and for samples following supplement only). No significant differences between groups for D and G.

here. However, they found that using 24-hour urine $\log_{10}(Eq/D)$ provided a clearer distinction of EqP status than absolute serum or urinary concentrations, claiming that the logarithm of the ratio was independent of isoflavone intake and minimized inter individual variation in pharmacokinetics [8]. In our pharmacokinetic study, ratios of Eq/D from serum samples did not distinguish between EqPs and EqNPs, while an absolute serum threshold of 22nM did, as long as samples were collected 12-24 hours post-ingestion (Figure 2).

Research protocols need to incorporate pharmacokinetic considerations. For example, one study examined urine collected 0-12 hours following a soy challenge [20]. Given that Eq does not increase in serum until after 12 hours, significant urine concentrations are unlikely before then. Surprisingly, some studies purporting to identify EqP do not report how status was assigned or the timing of isoflavone intake with respect to sampling [18,21]. Most studies collect fasting blood samples in the early morning, and isoflavone ingestion often occurs at breakfast (about 24 hours before sampling) or evening (about 12 hours before sampling). Thus the 12-24 hour post-ingestion window, during which serum Eq concentrations peak and accurate discrimination between EqP and EqNP populations can be made, is likely to be missed, reducing the probability of correct assessment of EqP status. If isoflavone consumption occurs primarily at dinner, the ability to assess EqP status from serum samples will be maximized by collecting samples mid-morning to midday. Observational and epidemiological studies can control for timing effects by using 24-hour dietary records and assessing whether conditions were met to assign EqP status: if isoflavone intake was insufficient in the 12-24 hours prior to sampling, then samples should be labeled unknown and removed from analyses comparing EqP and EqNP. If EqP status is of interest in epidemiological studies, participants should be instructed to consume soy at dinner, and then blood samples should be collected mid-day on the following day when possible. Alternatively, use of finger-prick dried blood samples, self-collected and sent by mail to researchers, can facilitate accurate assignment of EqP status by allowing participants to sample in optimal time windows following isoflavone ingestion and effectively administer a daidzein challenge test at home [14].

Further refinement of the guidelines presented here may be possible with increased sample sizes and sampling frequency as well as analysis and modeling of the effects of ethnicity [13]; age; sex [22]; and dietary differences including factors affecting isoflavone bioavailability [23] such as the form of isoflavones as aglycones in supplements versus conjugated forms in soy foods [12,24,25], dietary composition such as proportion of fat, carbohydrate, fiber, plant-protein [26,27], and fructooligosaccharides [28,29], and food matrix effects and gut transit time [12,30,31].

Conclusion

In epidemiological studies, evaluation of the health effects of isoflavones mediated by equol requires accurate assignment of EqP status. This in turn depends on whether sufficient daidzein substrate is present for conversion to equol and whether serum samples are collected in the 12-24 hour post-ingestion timeframe when equol concentrations peak. Studies presented here involved longer and higher supplementation than in most previous studies of 'long-term' isoflavone intake [10,22,32], and more time intensive sampling of the pharmacokinetic window in which Eq concentrations peak, and identified a threshold of 22nM for absolute serum equol concentrations as reliable for distinguishing between EqPs and EqNPs if samples are collected 12-24 hours following isoflavone ingestion. Further investigation of factors influencing intra- and inter-individual variation in equol pharmacokinetics may help optimize this guideline.

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