

Use of Physiologically Based Pharmacokinetic Modeling to Predict Human Gut Microbial Conversion of Daidzein to S-Equol

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ABSTRACT: A physiologically based pharmacokinetic (PBPK) model was developed for daidzein and its metabolite S-equol. Anaerobic in vitro incubations of pooled fecal samples from S-equol producers and nonproducers allowed definition of the kinetic constants. PBPK model-based predictions for the maximum daidzein plasma concentration (C_{max}) were comparable to literature data. The predictions also revealed that the C_{max} of S-equol in producers was only up to 0.22% that of daidzein, indicating that despite its higher estrogenicity, S-equol is likely to contribute to the overall estrogenicity upon human daidzein exposure to a only limited extent. An interspecies comparison between humans and rats revealed that the catalytic efficiency for S-equol formation in rats was 210-fold higher than that of human S-equol producers. The described in vitro–in silico strategy provides a proof-of-principle on how to include microbial metabolism in humans in PBPK modeling as part of the development of new approach methodologies (NAMs).

KEYWORDS: gut microbiota, daidzein, S-equol, physiologically based pharmacokinetic (PBPK) modeling

INTRODUCTION

The human body provides a habitat for vast microbial communities,¹ the majority of which reside in the gastrointestinal tract, especially the distal gut.² Through a wide range of biochemical reactions (e.g., hydrolysis, reduction, dehydroxylation, acetylation, and deacetylation), human gut microbiota may play a role in the toxicity of xenobiotics, changing their toxicokinetics and/or toxicodynamics.³ Though microbial composition and abundance vary among individuals, these differences do not necessarily translate to functional differences since the overall metabolic pathways of the gut microbiota appear to remain stable.⁴ However, this is not necessarily the case for the metabolism of food-borne xenobiotics as the consequences of intestinal microbial conversion for their effects on the host often remain to be quantified. This also holds for the gut microbial metabolism of daidzein.

Daidzein is a dietary isoflavone present in soy and soybean products that are structurally similar to the naturally occurring hormone 17 β -estradiol (E2) and thus referred to as a phytoestrogen. The consumption of isoflavones may have various health effects, such as increased bone mineral density, reduction of postmenopausal hot flashes, and antibreast cancer potentials.⁵ Upon gut microbial metabolism, daidzein yields dihydrodaidzein (DHD) as the intermediate metabolite, which can be subsequently converted to O-desmethylangolensin (O-DMA) or S-equol. The formation of S-equol only applies to S-equol producers (Figure 1).⁶

S-equol is reported to be more potent as an estrogen receptor (ER) agonist than its precursor daidzein,⁷ providing a potential for an influence of metabolism by the microbiota on the ultimate consequences of dietary exposure to daidzein. Therefore, it is also interesting to notice that there are

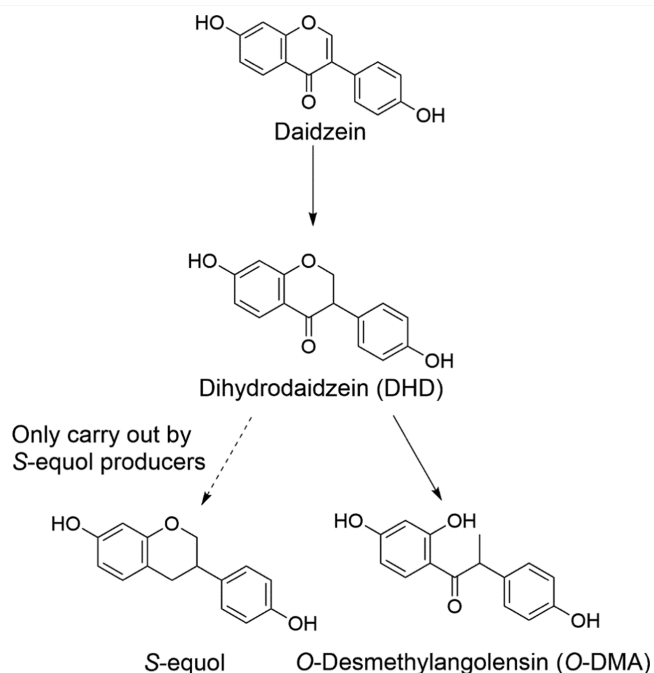


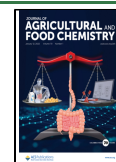
Figure 1. Metabolism of daidzein by gut microbiota in human. The plain arrows present the gut microbial conversion for both S-equol producers and nonproducers, while the dashed arrow presents the reaction only carried out by S-equol producers.

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interindividual differences in the potential for S-equol production, with around half of the Asian adult population and one-third of the Western adult population being S-equol producers.^{8,9} Diet, lifestyle, and genotype of the host are reported to affect the capacity for S-equol production,¹⁰ with the individual status of being an S-equol producer or nonproducers being relatively stable.¹¹ Given that S-equol producers make up a large part of the Asian population and the fact that their daily intake of isoflavone is relatively high,¹² they may be more susceptible to the estrogenic effects caused by S-equol. In a previous study,¹³ for rats, we used an in vitro–in silico approach including physiologically based pharmacokinetic (PBPK) modeling to study the impact of metabolism by the intestinal microbiota on the conversion of daidzein to S-equol and the resulting estrogenicity. This in vitro–in silico approach included quantification of the kinetics for microbial metabolite formation from daidzein and the development of a rat PBPK model that included gut microbial metabolism. The predictions revealed that daidzein is dominating the ER α -mediated estrogenicity even when taking the formation and estrogenicity of its more potent microbial metabolite S-equol into account. The in vitro–in silico approach presented a novel way of toxicity testing, enabling a shift from laboratory animal models to new approach methodologies (NAMs) based on in vitro and in silico approaches that could also be extended to human. In addition to ethical concerns, animal studies also may not adequately present the human situation given the potential kinetic and dynamic differences between animals and human, hampering extrapolation of data from experimental animals to the human situation.¹⁴ These considerations motivate the development of NAMs that combine human-based in vitro models with PBPK modeling to predict the human in vivo situation. The aim of the present study was to extend the PBPK model-based predictions for rats on the role of the gut microbiota in the in vivo effects of daidzein to human to allow predictions for the human situation and elucidate potential species differences.

To this end, human fecal samples of S-equol producers and nonproducers were used to derive kinetic parameters V_{\max} and K_m , which described daidzein gut microbial conversion to its metabolites DHD, S-equol (only for producers), and O-DMA. This enabled the inclusion of gut microbiota as an individual compartment in the PBPK model and facilitated the prediction of plasma concentrations of daidzein and S-equol in human.

MATERIALS AND METHODS

Materials and Reagents. Daidzein, S-equol, dimethyl sulfoxide (DMSO), glycerol, alamethicin, uridine 5'-diphosphoglucuronic acid (UDPGA), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), and tromethamine (Tris) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). DHD and O-DMA were purchased from Cayman Chemical (AA) and Plantech (Reading), respectively. Trifluoroacetic acid (TFA), MgCl₂, 37% HCl and NaOH were obtained from VWR (Amsterdam, The Netherlands).

Acetonitrile (ACN) and methanol were obtained from Biosolve BV (Valkenswaard, The Netherlands). Phosphate buffer saline (PBS) was supplied by Gibco (Paisley). Para-Pak SpinCon concentration system was bought from Meridian Bioscience (Schijndel, The Netherlands). Human pooled liver S9 fractions from 25 individuals (mixed gender) were supplied by Tebu-bio (Heerhugowaard, The Netherlands).

Anaerobic Incubations with Human Feces. Human fecal samples were collected from 15 volunteers. They were asked to fill out a short questionnaire and sign a consent form (Supporting Information 1) to confirm the participation and make sure they did not fall into any excluded class (e.g., pregnancy, use of antibiotics in

the past three months, etc.). The participation was anonymous, and researchers were not able to link the sample number with the participants. The design of the study was approved by the Medical Ethical Reviewing Committee of Wageningen University (METS-WU).

Each volunteer provided a one-time donation of around 5 g of feces. Feces were collected and weighed immediately upon donating. They were subsequently brought into an anaerobic chamber (Sheldon, Cornelius), containing 85% N₂, 10% CO₂, and 5% H₂, for further processing. The collected fecal samples were diluted five times (w/v) with an anaerobic solution consisting of 10% (v/v) glycerol in PBS. Subsequently, they were filtered using filter tubes and centrifuged at 2500g for 5 min. The resulting fecal suspension was well mixed, aliquoted, and stored at –80 °C until use.

A pretest for feces from each participant was carried out to distinguish S-equol producers from nonproducers. To this end, 100 μ L of incubation solutions were prepared containing (final concentrations) 60 mg/mL feces in PBS and 17.5 μ M daidzein that was added from a 200 times stock solution in DMSO. Samples were prepared in the anaerobic chamber and incubated in the above-mentioned anaerobic chamber at 37 °C for 8 h. Subsequently, 100 μ L of ice-cold methanol was added to each sample to stop the reaction, followed by a 10 min cooling on ice and a 15 min centrifugation at 21 500g at 4 °C. Supernatants were transferred into vials for liquid chromatography mass spectrometry (LC–MS) analysis. Feces samples from S-equol producers and nonproducers were mixed separately to get their respective pooled slurries.

The conditions for the anaerobic incubations with daidzein and human feces were adapted from those previously established for rat fecal incubations with some modifications.¹⁵ Final incubation conditions were 100 μ L prepared in anaerobic PBS containing 60 mg/mL pooled feces (v/v) (final concentration) from either S-equol producers or nonproducers, and daidzein (final concentration range 2.5–60 μ M) added from 200 times concentrated stock solutions in DMSO. Samples were prepared under the same anaerobic conditions as mentioned above. After incubating for 1 h in the anaerobic chamber at 37 °C, the reaction was terminated by adding 100 μ L of ice-cold methanol. Following a 10 min ice cooling and 15 min centrifugation at 21 500g at 4 °C, supernatants were transferred into vials for LC–MS analysis.

Blank controls were prepared by replacing daidzein with DMSO, and negative controls were prepared by replacing feces with PBS. Experiments were repeated three times.

Human Liver S9-Mediated Conjugation of S-Equol. Glucuronidation and sulfation of S-equol were carried out in incubations with pooled human liver S9 fractions performed as reported by Islam et al.,¹⁵ with some adjustments.

Glucuronidation was carried out in 100 μ L incubation mixtures, containing (final concentrations) 10 mM UDPGA, 0.025 mg/mL alamethicin, 10 mM MgCl₂, and 0.5 mg/mL human liver S9 protein in 50 mM Tris-HCl (pH 7.4) buffer.

After 1 min preincubation at 37 °C in a shaking water bath, the reactions were started by the addition of 1–200 μ M S-equol (added from 100 times concentrated stock solutions in DMSO). The incubations were carried out for 10 min until the addition of 25 μ L of ice-cold ACN to terminate the reactions. These conditions allowed a linear formation of S-equol glucuronides over time and with the S9 protein concentration. Following a 15 min centrifugation at 21 500g at 4 °C, supernatants were kept on ice until immediate ultra-performance liquid chromatography (UPLC) analysis. Blank incubations were performed in the absence of UDPGA, and negative incubations were performed without the addition of S-equol. Incubations for S-equol glucuronidation were repeated three times.

Sulfation of S-equol by human liver S9 was carried out by preparing 100 μ L incubation mixtures, containing 0.1 mM PAPS as a cofactor, 5 mM MgCl₂, and 1 mg/mL human liver S9 protein in 50 mM potassium phosphate (pH 7.4). The incubations were carried out for 60 min the same way as described above for the glucuronidation, except for the final concentrations of the substrate S-equol, which ranged from 0.5 to 100 μ M. Blank and negative incubations were

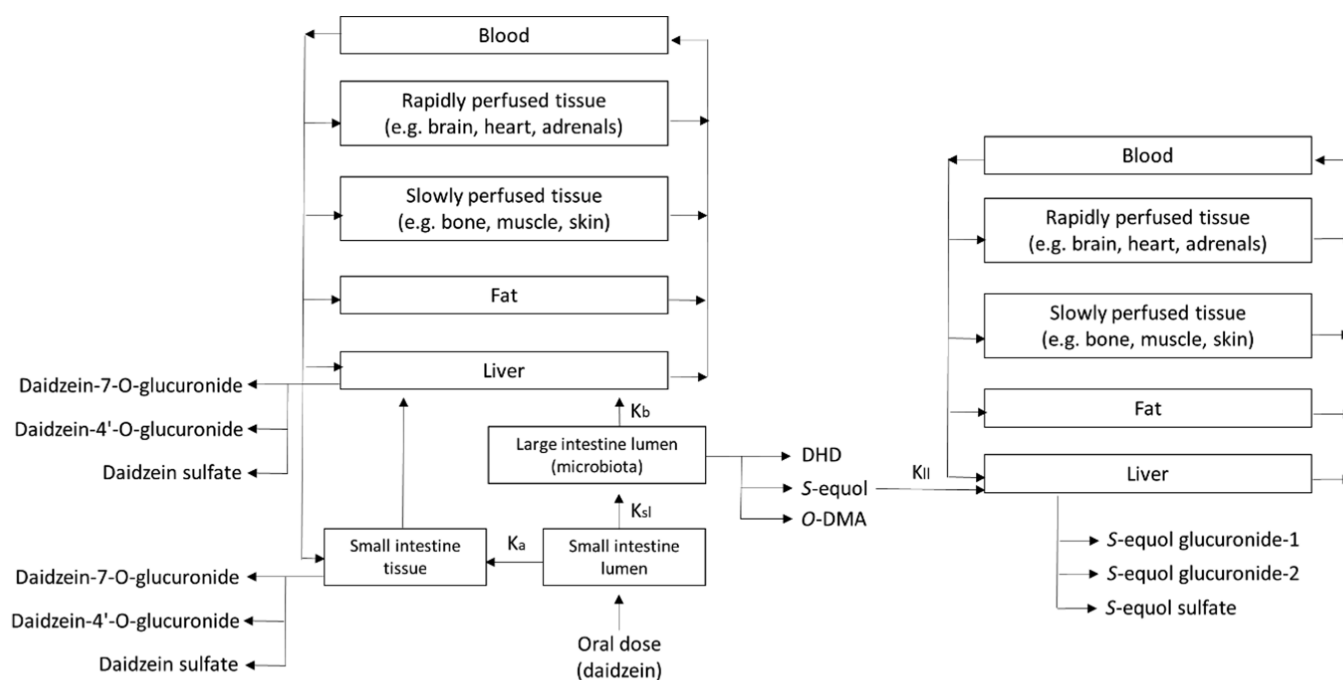


Figure 2. Structure of the PBPK model for daidzein with a submodel for S-equal. For S-equal nonproducers, only DHD and O-DMA are formed and the S-equal submodel is not applicable.

performed in the absence of PAPS and S-equal, respectively. Incubations for S-equal sulfation were repeated three times.

Quantification of S-Equal Glucuronide and Sulfate Conjugates. A UPLC system (Waters Acquity) (Etten-Leur, The Netherlands) was used to quantify the concentration of S-equal and its glucuronide and sulfate conjugates in human liver S9 incubations. The system was equipped with a guard column and a BEH C18 column (1.7 μm , 2.1 \times 50 mm, Waters), and a UV detector recording wavelengths of 190–320 nm were used.

Nanopure water with 0.1% TFA (v/v, solvent A) and ACN (solvent B) at a flow rate of 0.4 mL/min was used with the following gradients: 0% B for 0–0.2 min, 0–18% B for 0.20–0.40 min; 18% B for 0.20–3.00 min; 18–30% B for 3.00–3.50 min; 30–80% B for 3.50–5.00 min, 80–100% for 5.00–5.50 min, 100% B for 5.50–6.00, 100–0% B for 6.00–6.50 min, and 0% B for 6.50–7.00 min. The injection volume for each sample was 3.5 μL . Calibration curves were made for quantification of S-equal and its glucuronides and sulfates at a wavelength of 280 nm.

Quantification of Daidzein and Its Microbial Metabolites. A Shimadzu LC–MS/MS-8040 system (‘s-Hertogenbosch, The Netherlands) was used to quantify the concentration of daidzein and its metabolites in human fecal anaerobic incubations. The electrospray ionization (ESI) source and a Kinetex XB-C18 100A analytical column (1.7 μm , 100 \times 2.10 mm) were used for chemical ionization and compound separation, respectively. The mobile phase consisted of solvent A (0.1% TFA in nanopure water, v/v) and solvent B (0.1% TFA in ACN, v/v) at a flow rate of 0.3 mL/min, using the following mobile phase gradient program: 5% B for 0–1.00 min, 5–50% B for 1.00–1.50 min, 50–100% B for 1.50–4.50 min, 100% B for 4.50–6.60 min, 100–5% B for 6.60–10.50 min, and 5% B for 10.50–11.00 min. The column temperature was set at 40 $^{\circ}\text{C}$, and the injection volume for each sample was 10 μL . The flow of the drying gas (N_2) was 15 L/min and that of the nebulizing gas (Ar) was 2 L/min. The temperatures of desolvation line (DL) and heat block were set at 250 and 400 $^{\circ}\text{C}$, respectively. Data acquisition and processing were accomplished using Shimadzu LabSolutions LC/MS software (Kyoto, Japan).

Kinetic Analysis of the In Vitro Fecal or Liver S9 Incubations. The apparent maximum velocity (V_{max} , expressed in $\mu\text{mol}/(\text{h g})$ feces for fecal incubations or in $\mu\text{mol}/(\text{h mg})$ S9 protein for liver S9 incubations) and the apparent Michaelis–Menten

constant (K_m , expressed in μM) were obtained to describe the human gut microbial metabolite formation from daidzein, and the liver S9 catalyzed formation of S-equal glucuronide and sulfate metabolites. Data on the concentration-dependent rate of metabolite formation in human fecal anaerobic incubations with daidzein and in human liver S9 incubations with S-equal were fitted using GraphPad Prism 5.04 (GraphPad Software, CA) to the standard Michaelis–Menten equation

$$v = \frac{V_{\text{max}} \times [S]}{K_m + [S]}$$

where v and $[S]$ are the conversion rate (in $\mu\text{mol}/(\text{h g})$ feces or $\text{nmol}/(\text{min mg})$ S9 protein) and the substrate concentration (in μM), respectively. The V_{max} values thus obtained were scaled to the in vivo V_{max} values in the PBPK model as described in the next section.

PBPK Model Development. The human PBPK model was adapted from a rat model for daidzein containing a submodel for S-equal reported previously.¹³ As shown in Figure 2, the developed PBPK model includes separate compartments for blood, liver, fat, rapidly perfused tissue (e.g., heart, lung, and brain), slowly perfused tissue (e.g., skin, muscle, and bone), small intestine (lumen and tissue), and large intestine (lumen). The large intestine lumen compartment introduces the gut microbial activity in the model, allowing the description of microbial metabolite formation from daidzein. The model contains a submodel for S-equal to enable definition of systemic S-equal concentrations in S-equal producers.

The gut microbial metabolism of daidzein results in formation of an intermediate metabolite DHD, and two further metabolites O-DMA and S-equal, the latter only for S-equal producers. For S-equal producers, S-equal was modeled to form in the large intestine lumen and enter the liver with a rate constant of 4.56/h,¹⁶ while for S-equal nonproducers, only the main model for daidzein applies, which includes the formation of DHD and O-DMA from daidzein microbial conversion.¹⁷

Scaling of the kinetic V_{max} parameters obtained in vitro to the in vivo situation was included both for human fecal incubations and liver S9 incubations. For gut microbial conversions, the obtained apparent V_{max} values expressed in $\mu\text{mol}/(\text{h g})$ feces were scaled to the whole body using a fecal fraction of human body weight of 14 mL feces/kg bw.¹⁷ For liver and intestinal glucuronidation and sulfation of

daidzein, kinetic constants were taken from the published literature,¹⁵ and liver S9 incubations were performed for S-equal to define the kinetic constants for its conjugation. V_{\max} values (nmol/(min mg protein)) for S-equal glucuronidation and sulfation were scaled to in vivo V_{\max} values, using an S9 protein yield of 143 mg S9 protein/g tissue for the human liver. This value was obtained from the sum of 108 mg/g tissue and 35 mg/g tissue of cytosolic protein yield and microsomal protein yield for the human liver, respectively.¹⁸

Coding and integration of differential equations of the PBPK model were performed using Berkeley Madonna 8.3.18 (UC Berkeley, CA) and Rosenbrock's algorithms for stiff systems. The full model code can be found in the Supporting Information 2.

Sensitivity Analysis. To assess key parameters that have the largest influence on the predicted C_{\max} of daidzein and S-equal in S-equal producers, a sensitivity analysis was performed. Normalized sensitivity coefficients (SCs) were calculated based on the following equation¹⁹

$$SC = \frac{(C' - C)}{(P' - P)} \times \frac{P}{C}$$

in which P and P' are the initial and 5% increased parameter values, respectively; C represents the model output with an input of the initial parameter value, while C' represents the model output upon a 5% increase in the initial parameter value. When performing the sensitivity analysis, each input parameter was changed individually, while other parameters were maintained at their initial values. The larger a SC value, the larger the impact of that model parameter on the predicted C_{\max} of daidzein or S-equal.

RESULTS

Daidzein Metabolite Formation in Incubations with Individual Human Fecal Samples. Table 1 shows the

Table 1. Human Individual Microbial Formation of S-Equal After 8 h Anaerobic Fecal Incubation with Daidzein (17.5 μ M)

participant no.	production of S-equal	concentration of S-equal (μ M)
1	no	not detected (ND)
2	yes	1.75
3	no	ND
4	no	ND
5	no	ND
6	yes	3.61
7	yes	0.68
8	yes	1.32
9	no	ND
10	yes	0.27
11	no	ND
12	no	ND
13	yes	1.35
14	no	ND
15	no	ND

concentration of S-equal detected after 8 h anaerobic incubation of daidzein together with individual human fecal samples as a pretest to identify S-equal producers and nonproducers. Among 15 participants, 6 appeared to be S-equal producers and 9 to be nonproducers. For the S-equal producers, the gut microbial conversion of daidzein resulted in the formation of S-equal upon 8 h incubation at concentrations ranging from 0.268 to 3.611 μ M.

Kinetic Parameters for Daidzein Metabolite Formation by Pooled Human Fecal Samples. Subsequently, fecal samples from the 6 S-equal producers were pooled and incubated as mentioned above to obtain the kinetic constants V_{\max} and K_m for daidzein conversion to DHD, S-equal, and O-DMA, while feces from the 9 S-equal nonproducers were also pooled and incubated in the same way to obtain the kinetic constants V_{\max} and K_m for daidzein conversion to DHD and O-DMA. The formation of DHD, S-equal (only for S-equal producers), and O-DMA followed Michaelis–Menten kinetics, which allowed definition of their respective apparent V_{\max} , K_m , and catalytic efficiencies (calculated as V_{\max}/K_m). These values are presented in Table 2.

In fecal incubations with the sample from the S-equal producers, DHD was formed with the highest catalytic efficiency, which was 3.1- and 70-fold higher than that for S-equal and O-DMA formation, respectively. The catalytic efficiency for formation of DHD and O-DMA for S-equal producers was 1.23 and 0.4 times that obtained for the nonproducers.

Glucuronidation and Sulfation of S-Equal by Pooled Human Liver S9 Fractions. Kinetic parameters for the formation of S-equal glucuronides and sulfate were derived from in vitro incubations with pooled human liver S9 fractions. Figure 3 presents the S-equal concentration-dependent rates of metabolite formation for glucuronidation (Figure 3A) and sulfation (Figure 3B) that appeared to follow Michaelis–Menten kinetics. In incubations with PAPS, only formation of one S-equal sulfate metabolite was observed, for which substrate inhibition was observed at S-equal concentrations >25 μ M, a phenomenon frequently reported for sulfation reactions.^{20,21} For determination of the sulfation kinetic parameters, only data points of up to 25 μ M S-equal were included. V_{\max} , K_m , and catalytic efficiencies thus derived from the data in Figure 3 are presented in Table 3.

Formation of S-equal glucuronide-1 is observed with a scaled catalytic efficiency of 50.23 L/(h kg) bw, which is 11-fold higher than that for S-equal glucuronide-2 formation, with a value of 4.58 L/(h kg) bw. The formation of S-equal sulfate has a V_{\max} of 9.24 nmol/(min mg) S9 protein and a K_m of 6.50 μ M, resulting in a scaled catalytic efficiency of 313.46 L/(h kg) bw.

Model Evaluation by Comparison of Predictions to Literature Data. The kinetic constants obtained were scaled to the in vivo situation and integrated in the PBPK model to

Table 2. Kinetic Parameters for Formation of Daidzein Gut Microbial Metabolites

	S-equal producers			S-equal nonproducers	
	DHD	S-equal	O-DMA	DHD	O-DMA
V_{\max}^a (μ mol/(h g) feces)	0.024 \pm 0.004	0.009 \pm 0.001	0.001 \pm 0.0002	0.008 \pm 0.001	0.0007 \pm 0.0002
K_m^a (μ M)	6.24 \pm 3.45	7.24 \pm 3.24	18.07 \pm 8.20	2.55 \pm 1.95	5.12 \pm 4.31
catalytic efficiency ^b (mL/(h g) feces)	3.85	1.24	0.06	3.13	0.14

^aAverage \pm standard deviation (SD) of three independent experiments. ^bCalculated as $V_{\max}/K_m \times 1000$.

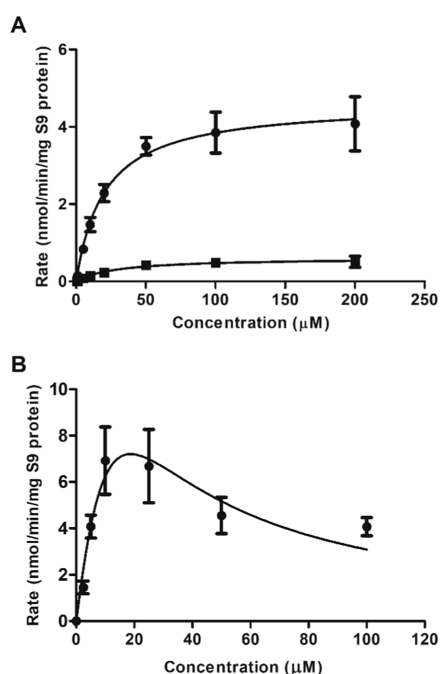


Figure 3. Concentration-dependent rate of formation of (A) S-equal glucuronides and (B) S-equal sulfate in incubations with pooled human liver S9 fractions. Data are presented as mean \pm SD of three independent experiments.

predict C_{\max} values for unconjugated daidzein and S-equal. To evaluate model performance, the model predicted C_{\max} of free daidzein was compared with human literature reporting C_{\max} upon oral administration of daidzein-containing food (e.g., soy milk, soy-based powder, etc.) upon oral doses in a range of 0.09–3.34 mg/kg bw daidzein.^{22–36} Literature data reported for the C_{\max} of daidzein (conjugated plus free) were multiplied by a factor of 2.08%³⁷ to estimate the C_{\max} for unconjugated daidzein in the circulation. Given that the blood-to-plasma ratio for neutral compounds is generally assumed to be 1,³⁸ no further correction between plasma and blood concentrations was applied. Figure 4 presents the ratio between the model predicted C_{\max} of unconjugated daidzein and the values derived from the data reported in literature. From this overview, it follows that there is a wide variability between data reported in literature. The model prediction is on average 1.62 times that of the reported in vivo plasma C_{\max} for daidzein.

Table 4 compares the PBPK model-based prediction of the cumulative 24 h urinary excretion of S-equal (mainly in the form of glucuronides and sulfates) with the in vivo S-equal urinary excretion, as reported in studies, with human volunteers orally exposed to daidzein.^{39,40} The overall ratio of the predicted versus the reported in vivo cumulative urinary

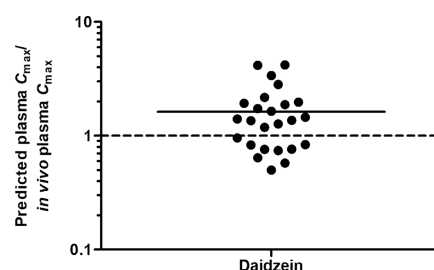


Figure 4. Ratio of predicted and in vivo observed C_{\max} of unconjugated daidzein upon daidzein administration of 0.09–3.34 mg/kg bw.^{22–36} Each data point represents a separate ratio.

Table 4. Ratio between Cumulative Urinary Excretion of S-Equal Predicted by the PBPK Model and as Reported in Studies with Human Volunteers^{39,40} 24 h upon Oral Doses of Daidzein

dose daidzein (mg/kg bw)	reported in vivo urinary excretion S-equal (mg)	model predicted urinary excretion S-equal (mg)	ratio predicted/in vivo urinary excretion
0.13	0.50	0.68	1.36
0.05	0.33	0.28	0.85
0.10	0.80	0.53	0.66
0.19	1.43	0.98	0.69
average			0.89

excretion amounts to 0.89 reveals that the model adequately predicts the formation of S-equal and its conjugated metabolites.

PBPK Model-Based Predictions of Human Plasma Profiles of S-Equal and Daidzein in Producers and Nonproducers. Subsequently, model-based predictions for the time-dependent human blood levels of S-equal and daidzein upon different oral dose levels were calculated for both S-equal producers and nonproducers (Figure 5). From these results, it follows that upon oral administration of 1 mg/kg bw daidzein in producers, S-equal was predicted to be present in plasma with a C_{\max} of 0.18 nM and an $AUC_{(0-4\text{ h})}$ of 2.02 nmol h/L. For its parent compound daidzein, a C_{\max} of 0.08 μ M and an $AUC_{(0-4\text{ h})}$ of 0.60 μ mol h/L were obtained, while in S-equal nonproducers, the daidzein C_{\max} and $AUC_{(0-4\text{ h})}$ were comparable at 0.09 μ M and 0.71 μ mol h/L, respectively. These results reveal that introducing gut microbiota as a separate compartment in the PBPK model provides a proof-of-concept for the effect of gut metabolism on systemic metabolite patterns in the host. The results presented in Figure 5 also reveal that the microbial intestinal daidzein conversion into S-equal appeared to be of only limited influence on the overall plasma daidzein kinetics, whereas S-equal levels are completely dependent on this reaction. The data also reveal

Table 3. Kinetic Parameters for the Human Liver S9-Mediated Conjugation of S-Equal

	S-equal glucuronide-1	S-equal glucuronide-2	S-equal sulfate
V_{\max}^a (nmol/(min mg) S9 protein)	4.62 \pm 0.19	0.61 \pm 0.05	9.24 \pm 1.51
K_m^a (μ M)	20.28 \pm 2.85	29.39 \pm 7.66	6.50 \pm 2.77
catalytic efficiency ^b (mL/(min mg) S9 protein)	0.23	0.02	1.42
scaled V_{\max}^c (μ mol/(h kg) bw)	1018.74	134.51	2037.48
scaled catalytic efficiency ^d (L/(h kg) bw)	50.23	4.58	313.46

^aAverage \pm SD of three independent experiments. ^bCalculated as V_{\max}/K_m . ^cScaled V_{\max} calculated from the in vitro V_{\max} based on an S9 protein yield of 143 mg S9 protein/g tissue for the human liver. ^dCalculated as scaled V_{\max}/K_m .

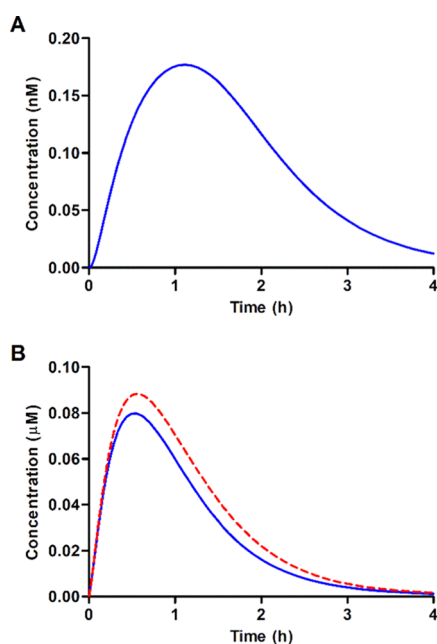


Figure 5. Model predictions for (A) S-equal and (B) daidzein plasma concentrations upon oral dosing of 1 mg/kg bw daidzein. The red dashed line is the model prediction of S-equal nonproducers and the solid blue line is that of S-equal producers.

that the S-equal C_{\max} values in S-equal producers are predicted to amount to only 0.22% of the plasma daidzein levels.

Sensitivity Analysis. A sensitivity analysis was performed to assess the most influential parameters affecting the C_{\max} of daidzein and S-equal. Oral doses of 1, 10, and 100 mg/kg bw daidzein, which are in the range of daily intake in Western diets, Asian diets, and soy supplementary diets,⁴¹ respectively, were applied in the analysis. Figure 6 shows the parameters having absolute normalized sensitivity coefficients (SCs) higher than 0.1 for at least one dose. From this, it follows that the predicted C_{\max} of daidzein is most sensitive to the fraction of blood flow to rapidly perfused tissue (Q_{Rc}), the fraction of liver (V_{Lc}), and liver S9 protein yield (V_{LS9}). The prediction of the C_{\max} of S-equal is predominantly influenced by Q_{Rc}, the maximum formation rate of S-equal by large intestine lumen (V_{maxLIEQUc}), and fraction of feces of body weight (V_{MB}). Compared to daidzein, the predicted C_{\max} of S-equal is more sensitive to large intestine related parameters, which refer to gut microbial metabolism.

Comparison between Human and Rat Microbial Metabolic Activities. To further characterize interspecies differences in daidzein metabolism by the gut microbiota, the metabolism of daidzein by the gut microbiota from human as quantified in the current study was compared to previous data obtained for rats.¹³ Figure 7 shows the daidzein concentration-dependent rates of metabolite formation for the two species, and kinetic parameter V_{\max} and K_m values are shown in Table 5. For the formation of S-equal, rat fecal samples showed a catalytic efficiency that was 209-fold higher than that obtained for human S-equal producers. For the formation of DHD and O-DMA, rat fecal samples also showed much higher catalytic efficiencies than human fecal samples, the values being, respectively, 54- and 118-fold higher in S-equal producers. These differences were due to substantially higher values for the apparent V_{\max} and somewhat lower K_m values.

DISCUSSION

The aim of the present study was to extend the *in vitro*–*in silico*-based NAM for prediction of *in vivo* daidzein metabolism previously developed for rats to human. The results obtained also allowed an interspecies comparison between rats and human.

The results obtained describe the kinetic parameters for human fecal metabolic conversion of daidzein by the human gut microbiota. These parameters were subsequently used to define a human PBPK model for daidzein that included metabolism by the intestinal microbiota. The formation of S-equal in some human individuals has been reported previously and is known to result from microbial metabolism of daidzein,^{42–44} although, in these earlier studies, kinetic parameters were not obtained since most of these studies focused on the isolation of bacterial strains capable of performing the conversions. Behr et al. reported that mammalian fecal materials are highly comparable to colonic ones in composition and function and can be used as a representative matrix to study the metabolic activity of the gut microbiota.⁴⁵ In addition, the use of anaerobic fecal incubations to define PBPK model kinetic constants for gut microbial metabolism of daidzein was previously shown to be valid for rats, for which PBPK model-based predictions made were in line with experimental data on C_{\max} levels for both daidzein and S-equal.¹³ Thus, to quantify PBPK model parameters for intestinal microbial metabolism, the use of anaerobic human fecal incubations appears to provide an adequate approach. This conclusion is corroborated by the present study because the PBPK model-based predictions for C_{\max} values of daidzein were, on average, 1.62-fold different from values actually reported in the literature. It should be noted that the literature data show wide variability while the model predicts an average value based on *in vitro* incubations of respective pooled human feces, so the actual differences between predicted and reported values may be influenced by interindividual differences and variation between reported studies.

PBPK model predictions were made for both S-equal producers and nonproducers. When comparing S-equal producers and nonproducers, S-equal producers show a slightly lower C_{\max} for daidzein than nonproducers, indicating that a small amount of daidzein undergoes the conversion to DHD and subsequently S-equal. However, since S-equal is known to be a more potent estrogen than its parent compound daidzein,^{46–48} it is of importance to study the estrogenic effects caused by S-equal taking its actual endogenous levels as compared to those of daidzein into account. As illustrated in Figure 5, the PBPK model predicted that upon oral administration of daidzein at 1 mg/kg bw, the C_{\max} of S-equal amounted to only 0.22% of that of daidzein in human, and this value was predicted to be 0.30% in rats.¹³ Considering the 12.7 times higher potency for S-equal than daidzein as derived from their EC₅₀ values in the ER-CALUX assay,¹³ it can be estimated that upon daidzein exposure, S-equal will contribute 2.80 and 3.81% to the overall estrogenicity compared with daidzein in human and rats, respectively. Thus, given the relatively low systemic concentrations in both species, S-equal is not expected to make a substantial contribution to the overall estrogenicity compared with its parent compound daidzein.

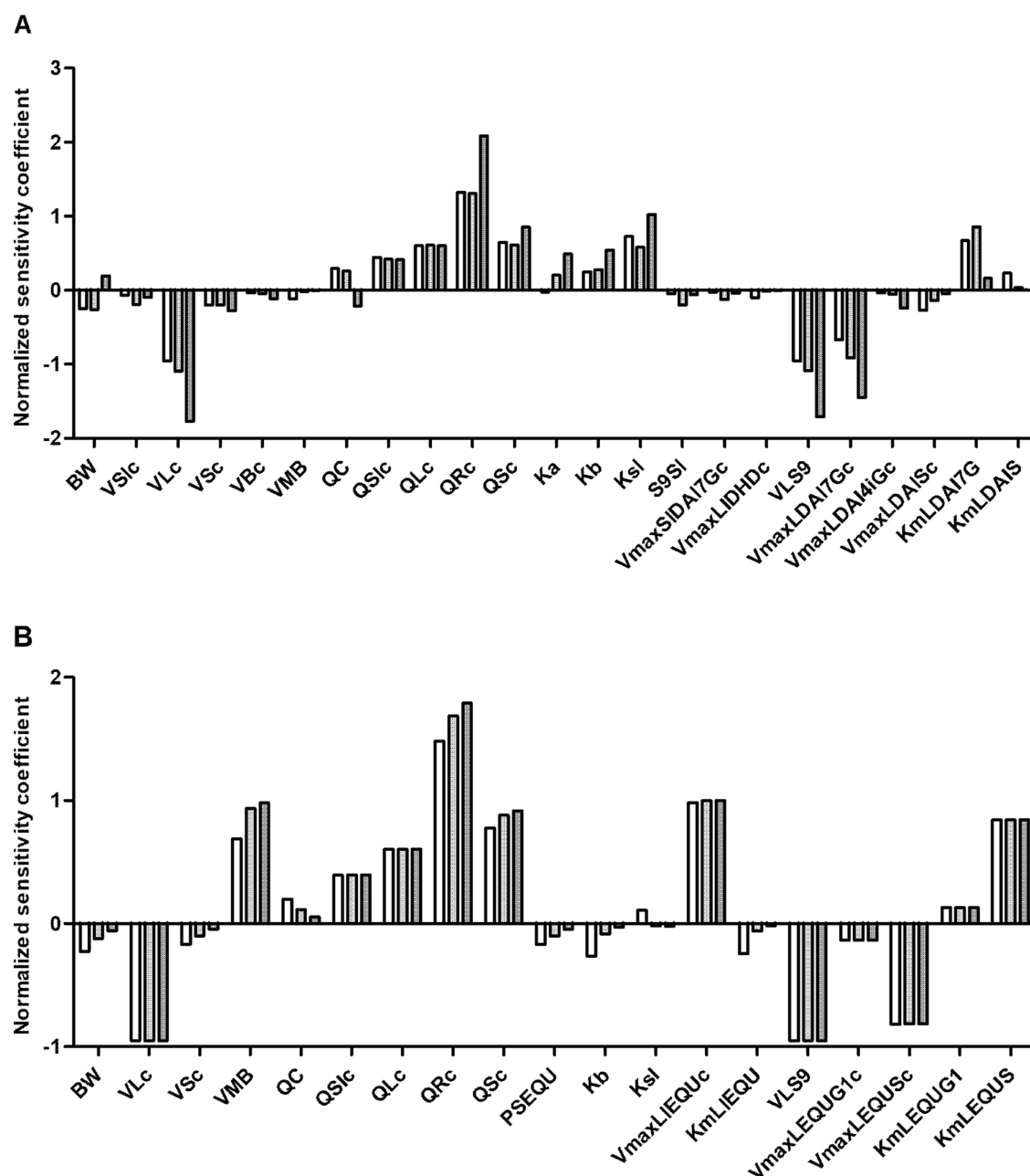


Figure 6. Sensitivity analysis for the predicted free C_{\max} of (A) daidzein and (B) S-equal at oral dose levels of 1 (white bars), 10 (light gray bars), and 100 (dark gray bars) mg/kg bw daidzein. Parameters represent the following: BW: body weight; VSIc: fraction of small intestine; VLC: fraction of liver; VSc: fraction of slowly perfused tissue; VBC: fraction of blood; VMB: fraction of feces of body weight; QC: cardiac output; QSIc: fraction of blood flow to small intestine; QLC: fraction of blood flow to liver; QRC: fraction of blood flow to rapidly perfused tissue; QSc: fraction of blood flow to slowly perfused tissue; PSDAI: slowly perfused tissue/blood partition coefficient of daidzein; Ka: absorption rate of daidzein to intestinal tissue; Kb: transfer rate of daidzein from large intestinal lumen to liver; Ksl: transfer rate of daidzein to feces; S9SI: small intestinal S9 protein yield; VmaxSIDAI7Gc: V_{\max} for formation of daidzein-7-O-glucuronide by small intestine; VLS9: liver S9 protein yield; VmaxLIDHDc: V_{\max} for formation of DHD by large intestine lumen; VmaxLDAI7Gc: V_{\max} for formation of daidzein-7-O-glucuronide by liver; VmaxLDAI4Gc: V_{\max} for formation of daidzein-4'-O-glucuronide by liver; VmaxLDAISc: V_{\max} for formation of daidzein-sulfate by liver; KmLDAI7G: K_m for formation of daidzein-7-O-glucuronide by liver; KmLDAIS: K_m for formation of daidzein-sulfate by liver; PSEQU: slowly perfused tissue/blood partition coefficient of S-equal; VmaxLIEQUc: V_{\max} for formation of S-equal by large intestine lumen; KmLIEQU: K_m for formation of S-equal by large intestine lumen; VmaxLEQU1c: V_{\max} for formation of S-equal glucuronide-1 by liver; VmaxLEQUsc: V_{\max} for formation of S-equal sulfate by liver; KmLEQU1: K_m for formation of S-equal glucuronide-1 by liver; KmLEQUsc: K_m for formation of S-equal sulfate by liver.

Catalytic efficiencies for the formation of DHD and O-DMA in S-equal producers were 1.23- and 0.4-fold those obtained for nonproducers. This indicates that the conversion from daidzein to DHD appears comparable in the two populations, with the subsequent conversion of DHD to O-DMA being somewhat less effective in S-equal producers, who also convert

DHD to S-equal. The reason underlying the lack of S-equal production and its correlation with the composition and abundance of microbiota remains to be elucidated.^{49,50} Further studies on the human individual microbiota density and composition between S-equal producers and nonproducers may provide new clues for the observed differences.

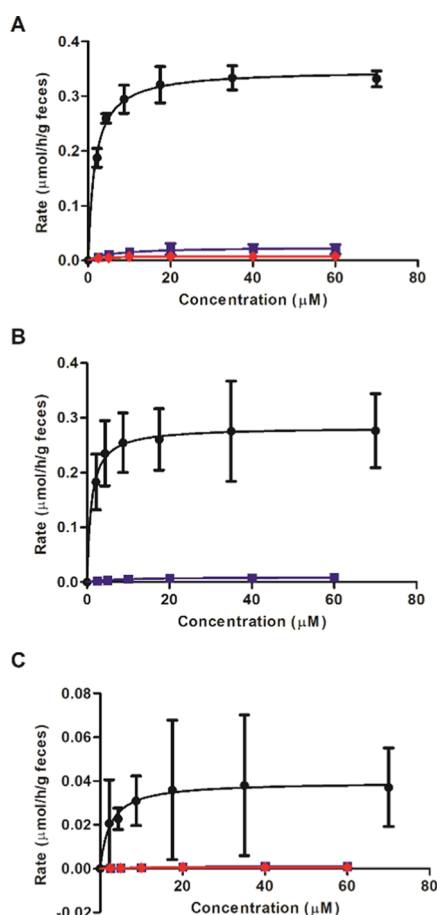


Figure 7. Comparison of microbial formation of (A) DHD, (B) S-equal, and (C) O-DMA in rats (black circles), human S-equal producers (blue squares) and nonproducers (red rhombus) in fecal incubations with daidzein. Data are presented as mean \pm SD of three independent experiments.

The results of the present study also enabled a comparison of the gut microbial conversion of daidzein in human to that in rats. This revealed that for the formation of S-equal, rat feces has a catalytic efficiency that is 209-fold higher than that of feces from pooled human S-equal producers. For formation of DHD and O-DMA, rat feces also shows substantially higher catalytic efficiencies being 54- and 118-fold higher than that of human. Taking into account that rat feces make up around 5% of the body weight, while for human, that value is only 1.4%,¹⁷ the differences in per kg bw basis are even larger, adding an extra 3.57-fold interspecies difference to the differences between rat and human for the formation of each metabolite.

Contribution of S-equal to the overall estrogenicity in these two species can also be further compared. Rodents are

reported to be S-equal producers,^{51,52} while in human, only 20–55% of the population are S-equal producers.^{8,9} The results of the current work, however, reveal that in spite of this species difference, rats may still reflect the human situation because the results of the PBPK modeling predict that S-equal contributes to the overall estrogenicity upon exposure to daidzein to a only limited extent in both species.

In the present study, an in vitro approach using human fecal material was developed to derive kinetics for a PBPK model, which included microbiota as a separate compartment. The possibility of describing gut microbial conversions provides a unique tool to predict plasma concentrations of daidzein and S-equal for different target groups. Taken all together, the described in vitro–in silico strategy provides a proof-of-principle on how to include metabolism by the gut microbiota in the PBPK model for human as part of the development of NAMs in safety testing.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c03950>.

Supporting information 1: Questionnaire and consent form for human fecal sample collection; supporting information 2: PBPK model code (ZIP)

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Table 5. Comparison of Kinetic Parameters between Human and Rat for the Formation of Daidzein Microbial Metabolites

	DHD			S-equal			O-DMA	
	rats	human S-equal producers	human S-equal nonproducers	rats	human S-equal producers	rats	human S-equal producers	human S-equal nonproducers
V_{\max}^a ($\mu\text{mol}/(\text{h g feces})$)	0.35 \pm 0.01	0.024 \pm 0.004	0.008 \pm 0.001	0.28 \pm 0.02	0.009 \pm 0.001	0.04 \pm 0.01	0.001 \pm 0.0002	0.001 \pm 0.0002
K_m^a (μM)	1.69 \pm 0.21	6.24 \pm 3.45	2.55 \pm 1.95	1.08 \pm 0.55	7.24 \pm 3.24	2.42 \pm 2.31	5.12 \pm 4.31	18.07 \pm 8.20
catalytic efficiency ^b ($\text{mL}/(\text{h g feces})$)	207.10	3.85	3.13	259.26	1.24	16.53	0.14	0.06

^aAverage \pm SD of three independent experiments. ^bCalculated as V_{\max}/K_m .

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Notes

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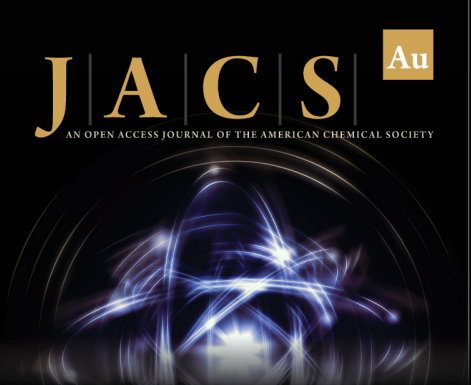
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