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Estrogenic activity of capsule coffee using the VM7Luc4E2 assay

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ABSTRACT

Coffee brewed from capsule machines may contain estrogenic chemicals migrated from plastic, but the estrogenic activity of capsule coffee has not been evaluated. This study evaluated the estrogenic activity of capsule coffee using the VM7Luc4E2 estrogen receptor transcriptional activation assay. Estrogenic potentials of six capsule coffee samples were calculated using relative maximum amplitude response of E2 (>15%RME2 indicative of estrogenic activity) and estradiol equivalent factor (EEF). Estrogenic chemical content was determined using ultra-performance liquid chromatography with tandem mass spectrometry. All capsule coffee samples possessed estrogenic activity (48–56%RME2). EEFs were 6–7 orders of magnitude lower than that of E2, ($1.2 \times 10^{-7} - 1.7 \times 10^{-6}$), indicating substantially weaker estrogenic potencies. Bisphenol A, bisphenol F, benzophenone, 4-nonylphenol, dibutyl phthalate, and dimethyl terephthalate were detected in capsule coffee. Capsule coffee exhibited estrogenic activity in vitro, and its estrogenic chemical content is likely driving its estrogenicity, warranting further investigations to fully understand the degree to which they are related and to predict the estrogenic potential based on the concentration of estrogenic chemicals.

1. Introduction

Coffee is among the most popular beverages consumed globally and particularly in the US. Nearly half of American adults drink coffee daily and more adults reported drinking coffee than water during the previous day (Loftfield et al., 2016; Association, 2016), signifying the ubiquity and habitual nature of coffee consumption. The most common method of brewing coffee in the US is via a drip coffee maker (50% of all coffee) but single-serving brewers (28%), which typically utilize a pre-packaged plastic capsule (henceforth referred to as capsule coffee), are becoming popular and are the second most common method (Association, 2016). Capsule coffee offers convenience and reliability as they are quick to brew, require little clean-up, and contain a consistent amount of coffee grounds between capsules.

However, capsule coffee consumption may increase exposure to harmful estrogenic chemicals (Sakaki et al., 2020). In the production of plastic, monomers, polymers and additives such as plasticizers, many of which have estrogenic activity (Yang et al., 2011; Bittner et al., 2014), are polymerized, forming a base resin, such as in the case of a bisphenol A (BPA)-based resin. This is often further mixed with other resins and additives to improve the properties or quality of the plastic. Estrogenic chemicals may leach from the finished product because the polymerization may be incomplete, leaving residual monomers and additives, or because the additives may not be chemically bound to the polymer (Yang et al., 2011; Bittner et al., 2014; Le et al., 2008). These chemicals have been shown to migrate from food packaging and containers into food (Fasano et al., 2012). Heat can also potentiate the migration, as BPA was found to migrate from a plastic water bottle at rates up to 55 times greater with boiling water compared to room temperature water (Le et al., 2008). Brewing capsule coffee typically involves forcing hot water through a plastic capsule filled with coffee grounds which may cause migration of these chemicals into the coffee. Previous studies have indeed detected contamination of estrogenic chemicals in capsule coffee, including dibutyl phthalate (DBP) (2.5–70 μg/L), bis(2-ethylhexyl) phthalate (DEHP) (3.3–1560 μg/L) and diethyl adipate (25.1 μg/L) (Sakaki et al.,

Abbreviations: 4-NP, 4-nonylphenol; BP, benzophenone; BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; DBP, dibutyl phthalate; E2, 17β-estradiol; EEF, estradiol equivalent factor; EEQ, estradiol equivalent concentration; HPLC, high-performance liquid chromatography; RLU, relative luminescence units; RME2, relative maximum amplitude response of E2; UPLC-MS/MS, ultra-performance liquid chromatography with tandem mass spectrometry.

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2020; Di Bella et al., 2014; De Toni et al., 2017), although estrogenic activity was not evaluated.

Exogenous estrogenic chemicals are chemicals that mimic the actions of estrogen hormones such as 17β-estradiol and are considered to possess estrogenic activity. Exposure to these chemicals has been shown to adversely affect organs involved in reproduction including the ovary, uterus, vagina and anterior pituitary gland, leading to endometriosis, polycystic ovary syndrome, infertility or abnormal pregnancy, prostate cancer, thyroid cancer and developmental toxicity (Kortenkamp et al., 2011; Gore et al., 2015). Due to concerns over toxicity and damage to endocrine and reproductive systems, tolerable daily intake guidelines have been established for numerous estrogenic chemicals including BPA, bisphenol F (BPF), benzophenone (BP), 4-nonylphenol (4-NP) and DBP (Nielsen et al., 2000; EFSA, 2015, 2017; European Food Safety Authority, 2005; Zoller et al., 2016).

To our knowledge, no study has attempted to evaluate the degree of estrogenic activity of capsule coffee in vitro. This is an important step in assessing the potential for adverse health effects of consuming capsule coffee. Therefore, the objective of this exploratory study was to determine the estrogenic activity of capsule coffee and relate it to estrogenic chemical content.

2. Materials and Methods

2.1. Capsule coffee samples and preparation

Six brands of capsule coffee were selected and prepared. Capsules 1–4 were only compatible with one brewing machine and capsules 5 and 6 were only compatible with another brewing machine. Each brewing machine produced one blank (Blank 1 corresponds to the brewing machine used for capsules 1–4 and blank 2 corresponds to the brewing machine used for capsules 5 and 6). Details on the coffee bean type, roast, weight of the coffee grinds and volume dispensed are presented in S1 Table. Capsule coffee and brewing machines were purchased through online retail stores.

The coffee samples were brewed as follows: first, the capsule coffee brewing machine was run three times using only high-performance liquid chromatography (HPLC)-grade water (Fisher Scientific, Fair Lawn, NJ) without a capsule to rinse any residue. Then, a coffee capsule or blank was brewed in triplicate using the smallest volume setting with HPLC-grade water, each time replacing the capsule with a new one. All three runs were combined. This process was repeated for each of the six brands of capsule coffee.

2.2. Extraction procedure for estrogenic activity

To create extracts from the brewed coffee, 25 mL of each coffee sample was first centrifuged at 2500 rpm for 5 min to eliminate the solids. Then, the supernatant was evaporated to dryness using an automated evaporator (EZ-2 Genevac Evaporator, Genevac Ltd, Ipswich, United Kingdom). The temperature was held at 25^{0}C and pressure was gradually reduced from 993 mbar to 2 mbar based on the 60-min low boiling point parameters. Finally, the dried sample was reconstituted with 0.1 mL 100% methanol (Sigma-Aldrich, Inc., Darmstadt, Germany).

2.3. VM7Luc4E2 assay for the determination of estrogenic activity

The VM7Luc4E2 (previously named BG1Luc4E2) transcriptional activation test method (Brennan et al., 2016) identifies substances with estrogen receptor agonist or antagonist activity via induction of firefly luciferase (Rogers and Denison, 2000) and has demonstrated high degrees of accuracy and reliability in screening these substances in vitro (U.S. Environmental Protection Agency, 2009; Interagency Coordinating Committee on the Validation of Alternative Methods). It has been approved as a screening method for estrogenic chemicals by the Organization for Economic Co-operation and Development, the Environmental Protection Agency, the Interagency Coordinating Committee on the Validation of Alternative Methods and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (Antagonists, 2012). This method uses VM7 cells, a variant of breast cancer-derived MCF-7 cells, a cell line stably transfected with an estrogen-responsive luminescence gene, to measure estrogen receptor-mediated transcriptional activity (Interagency Coordinating Committee on the Validation of Alternative Methods). Cells were grown according to a slightly modified version of a previous protocol (Bittner et al., 2014). In short, cells were maintained in cell culture medium consisting of phenol red-free Dulbecco's Modified Eagle Medium with 8% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin, L-glutamine and sodium pyruvate. Prior to assaying for estrogenic activity, cells were placed in an estrogen-free medium modified from the cell culture medium (phenol red-free containing Dulbecco's Modified Eagle Medium with 4.5% charcoal-stripped fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin, L-glutamine and sodium pyruvate) for 3 days. Then, cells were seeded at 5,000 cells per well in 25 μL of estrogen-free medium in 384-well plates for 20 h, followed by incubation for 20 h with test extracts with four independent trials. There was no significant increase in the number of cells after the short treatment by estrogenic compounds. Therefore, the increased luminescence is primarily due to the luciferase expression in individual cells.

Cell cytotoxicity was measured microscopically (Yang et al., 2011; Bittner et al., 2014) by altered cell morphology score parameters: 1 = normal cell morphology, 2 = low cytotoxicity (10–50% cells with altered morphology), 3 = moderate cytotoxicity (50–90% of cells with altered morphology), 4 = high cytotoxicity (few or no visible cells). A score of 2 or higher was indicative of cytotoxicity. Cytotoxicity in this assay is unlikely to cause false positives. Estrogen-free medium was then aspirated, cells were lysed with lysis buffer (cat. #A1731, Promega, Madison, WI, USA), and luciferase expression was measured in an automated microplate luminometer (Tristar Berthold Technology, Germany) with the Promega Luciferase Assay System (Promega, Madison, WI, USA) following the manufacturer's protocol. Cells were obtained from Dr. Mike Denison at the University of California at Davis.

2.4. Calculation of estrogenic activity and potential

Estrogenic activity of coffee extracts was calculated as percent relative maximum of E2 (%RME2), the response amplitude of the sample (in relative luminescence units (RLU)) relative to that induced by 17β-estradiol (E2, positive control), and corrected for the background response to the vehicle control. (Eq. (1)) (Bittner et al., 2014). Ethanol (1%) was used as the vehicle control in the testing plates and was also presented in testing concentrations and testing samples including blanks.

$$\%\mathbf{RME2} = 100\%\mathbf{x}\frac{\mathbf{MaxRLU_{sample}} - \mathbf{RLU_{vehiclecontrol}}}{\mathbf{MaxRLU_{E2}} - \mathbf{RLU_{vehiclecontrol}}} \quad (1)$$

The %RME2 was set to 100% for E2 and 0% for vehicle control. Samples were deemed to have estrogenic activity if > 15%RME2, three times the standard deviation of the vehicle control and therefore a conservative measure of estrogenic activity detectability. Aliquots of the highest concentration of E2, blanks and capsule coffee samples were diluted to produce ten different concentrations. The ten concentrations were tested for capsule coffee extracts (approximately log-2 to log2 μg/mL) and the positive control E2 (log-13.28 to log-9.7 μg/mL) (see S2 Table for concentrations in μg/ml units). The blank water samples were concentrated using the same method as for the other test samples, and the precipitants (if present) were also diluted in the same method as for the other test samples. The stimulation of luciferase

expression was confirmed to be estrogenic by suppression of estrogenic activity with co-incubation with 0.1 μM fulvestrant (ICI 182,780), an estrogen receptor down-regulator (anti-estrogen control) (cat. # 1047, CAS 129453–61-8, Tocris, Bio-Techne, Minneapolis, MN). Test substances with at least two data points > 15%RME2 were compared with the data points of the test substance with ICI 182,780 at the same log concentrations. Differences of at least 30%RME2 (i.e. ICI 182,780 suppression of at least 30%RME2) at p-value < 0.05 (paired t-test) were considered statistically significant (Bittner et al., 2014). Concentration-response curves were created using the mean values of quadruplicate runs for each concentration of E2, blanks and coffee samples. E_{max} was defined as the highest %RME2 value for each substance/sample.

Estrogenic potential of capsule coffee samples relative to that of E2 was calculated using estradiol equivalent factor (EEF). EEF is a measure of a substance or sample's estrogenic potency relative to that of E2 and is calculated as the concentration of E2 at which half of the maximum response is observed (EC_{50}) divided by that of the sample (Eq. (2)).

$$\mathbf{EEF} = \frac{\mathbf{EC50_{E2}}}{\mathbf{EC50_{sample}}} \quad (2)$$

Mean %RME2 values for each of the ten concentrations of E2 and capsule coffee samples were used in a nonlinear regression curve fitting model (log(agonist) vs. response, GraphPad Prism version 5.00, GraphPad Software, San Diego, California, USA) to obtain EC_{50} values. The calculated estradiol equivalent (EEQ), a measure of estrogenic potency of a compound, of each capsule coffee was determined by multiplying the EEF value by its concentration of coffee grounds in water used in brewing (Eq. (3)).

$$\mathbf{EEQ} = \mathbf{EEFxConcentration} \quad (3)$$

2.5. Extraction, identification and quantification of estrogenic chemicals in capsule coffee

Preparation of the capsule coffee samples for the analysis of estrogenic chemical content were as follows: first, 25 mL of capsule coffee was spiked with 50 μL of the surrogate quality control analyte 4-hydroxy-biphenyl-d9 (CDN Isotopes, Pointe-Claire, Quebec, Canada) due to its structural similarities with bisphenols and its minimal possibility to be naturally occurring in the samples. The lab control sample and matrix spike sample were spiked with the analytical standards of the target estrogenic chemicals to a final concentration of 500 ng/mL for each estrogenic chemical. The analytical standards were purchased from Sigma-Aldrich and included BPF (cat. # 51453-100MG, CAS 620-92-8), BPA (cat. # 239658-50G, CAS 80-05-7), bisphenol S (BPS) (cat. # 43034-100MG, CAS 80-09-1), 4-NP (cat. # 46405-100MG, CAS 104-40-5), dimethyl terephthalate (DMTP) (cat. # 185124-500G, CAS 120-61-6), DEHP (cat.# 80030-1ML, CAS 117-81-7), DBP (cat. # 524980-25ML, CAS 84-74-2), epsilon-caprolactam (cat. # C2204-250G, 105-60-2) and BP (cat. # 427551-1G, CAS 119-61-9). Then, 20 mL ethyl acetate (Fisher Scientific, Fair Lawn, NJ) was added and the resulting mixture was vortexed and then centrifuged at 2500 rpm for 5 min. After centrifugation, the top ethyl acetate layer was removed and the remaining emulsion was evaporated utilizing a Genevac automated evaporator on the low boiling point setting for 60 min. Finally, 950 μL of methanol and 50 μL of the internal standard 1-napthol-d3 were added and mixed. Extra care was taken to limit contact with plastic during the preparation and handling processes. Glass equipment including syringes, vials and pipettes were used, and plastic caps were replaced with tightly wrapped aluminum foil. The mixture was analyzed using an ultra-performance liquid chromatograph coupled with tandem mass spectrometry (UPLC-MS/MS). The identification and quantification of unknown estrogenic chemicals were conducted based on retention time and m/z values of compounds or signature ion fragments of a peak generated by the estrogenic chemical standards.

2.6. UPLC and mass spectrometry conditions

Quality control and coffee samples were analyzed using a Waters Acquity™ UPLC® coupled with an Acquity™ TQD™ tandem mass spectrometer (Waters Co., Milford, MA), with analytic conditions modified from Langer et al. (Langer et al., 2014) (S3 Table). The detection and quantification of analytes, surrogate, and internal standard compounds were performed in negative ESI-MS/MS mode (MRM) using the Waters, Inc. IntelliStart™ software for analyte signal optimization. Statistical analyses for obtaining calibration and quantification results for all compounds were performed using Waters QuanLynx™, which was included in the MassLynx software v.4.2. Parameters for the mass spectrometer were set as follows: capillary voltage, 3.2 kV; variable cone voltage and collision energy; desolvation temperature, 350 °C; source temperature, 145 °C; desolvation gas flow, 600 L/h; collision gas flow, 0.2 mL/min.

2.7. Method validation

The UPLC-MS/MS analytical method was tested for its validity in determining estrogenic chemical content in samples. Four independent trials were conducted for precision and accuracy, and seven independent trials were conducted for the method detection limit. This method was highly accurate, (overall recovery = 94.9%), precise (overall precision = 2.0%) and sensitive (S4 Table). Thus, it was suitable for detecting and quantifying the estrogenic chemical content in capsule coffee.

3. Results

3.1. Estrogenic activity of capsule coffee

Concentration-response curves from the VM7Luc4E2 assay quantifying estrogenic activity of coffee extracts are given in Fig. 1. Using 15%RME2 as the cut-off for determining estrogenic activity, E2 and all capsule coffee extracts were positive for estrogenic activity while blanks did not have estrogenic activity. Statistically significant inhibition of estrogenic activity by co-incubation with ICI 182,780 confirmed that estrogenic activity was specifically due to agonistic activity of estrogen receptors (all p-values < 0.05).

E_{max}, EC_{50}, EEF and EEQ values are displayed in Table 1. E_{max} for the positive control, E2, was set to 100%, and E_{max} for capsule coffee samples ranged 48–56%RME2, indicating that the maximum efficacies of the capsule coffee samples were approximately half of that of E2. The concentrations required to reach the half-maximal response (EC_{50}) for capsule coffee ranged 0.3209–0.6818 μg/mL, which were approximately seven orders of magnitude higher than that of E2. The potencies of capsule coffees for inducing estrogenic activity were substantially weaker than the potency of E2, ranging 1.2×10^{-7} to 1.1×10^{-6} EEF. Capsule 2 had both the highest estrogenic potency (EEF = 8.0×10^{-7}) and the highest expected estradiol equivalent load (EEQ = 102.88 ng/mL).

3.2. Identification and quantification of estrogenic chemicals

The contents of estrogenic chemicals in capsule coffee samples are displayed in Table 2. BPA, BPF, BP, 4-NP, and DBP were detected while caprolactam, DEHP, DMTP and BPS were not detected in the capsule coffee samples. Among all of the estrogenic chemicals analyzed, BP was the most common (4/6 capsule coffee samples), ranging in concentrations of 30.51–149.40 ng/mL brewed coffee. DBP was the second most common (3/6), and BPA, BPF and 4-NP were each detected in two capsule coffee samples. Of the four capsules brewed

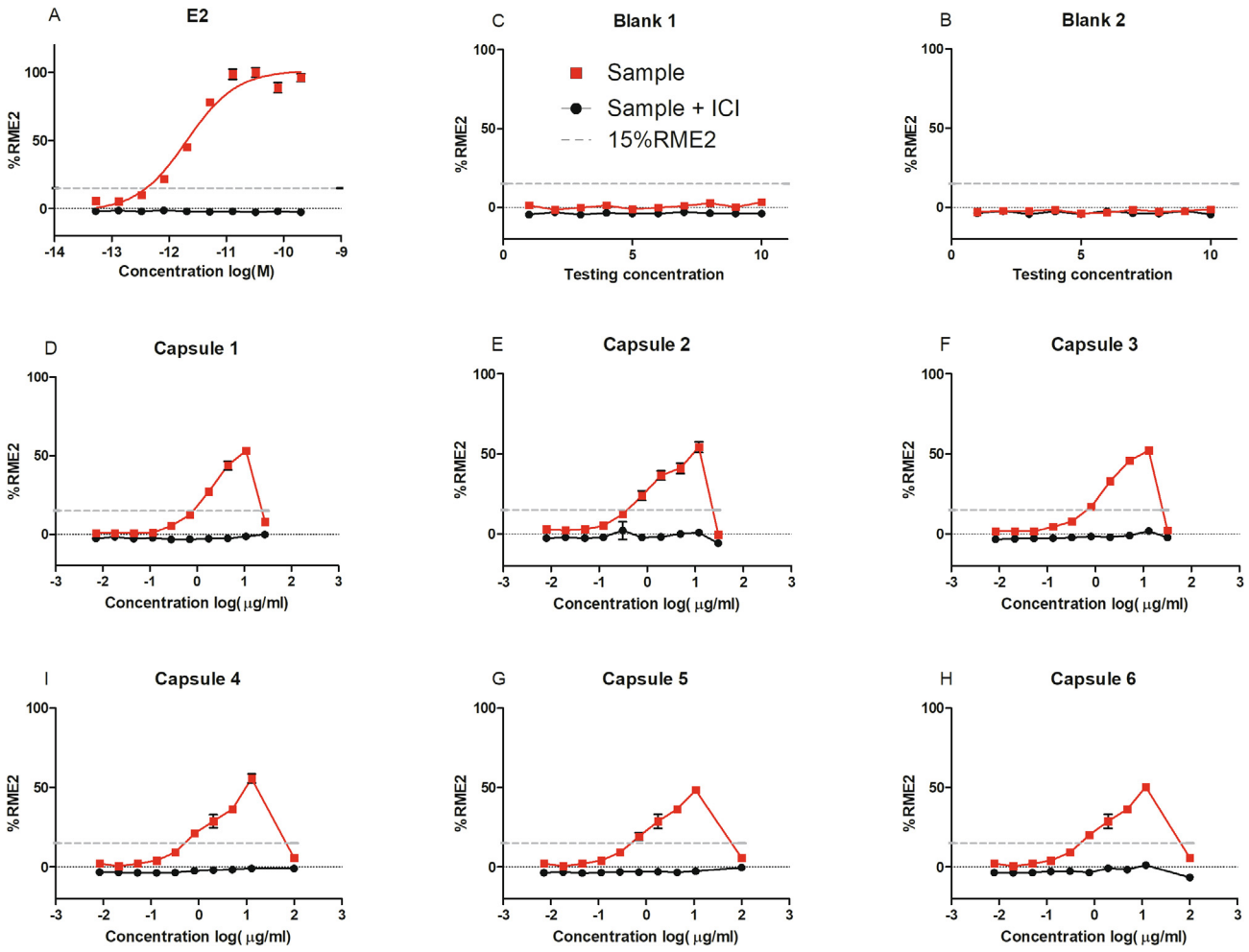

Fig. 1. VM7Luc4E2 assay of estrogenic activity of capsule coffee. Data are concentration–response curves with log concentration (M or μg/mL) on the x-axis and the percent relative maximum amplitude of E2 (%RME2) on the y-axis. For blank samples, the x-axis indicates the testing concentration as determined using the same method as for the other test samples, with 10 as the lowest concentration and 1 as the highest concentration. Maximum %RME2 of E2 (positive control, 17β-estradiol) is set to 100% and the normalized vehicle control is set to 0%. Vehicle control + 3 standard deviations is the normalized estrogenic activity value for 3 standard deviations of the vehicle control for the particular run (15% RME2) and is used as the cut-off to determine whether a substance is positive for estrogenic activity. Estrogenic activity was confirmed by lack of estrogenic activity induced by co-incubation with the inhibitor ICI 182,780. Each data point represents the normalized estrogenic activity of quadruplicate tests at each of the ten concentrations. Error bars indicate standard error of the mean and are often smaller than the space allocated by the data points. Blank 1 and capsules 1–4 were used in one brand of capsule coffee brewing machine and blank 2 and capsules 5–6 were used in a second brand. E2, positive control (17β-estradiol); ICI, anti-estrogen control ICI 182,780.

Table 1
Concentration (of coffee grounds), E_{max}, EC_{50}, EEF and EEQ values of capsule coffee.

Samples	Conc. (g/mL)	E_{max} [a] (%RME2)	EC_{50}^{b} (M)	EC_{50}^{b} (μg/mL)	EEF[c]	EEQ[d] (ng/mL)
E2		100	2.0×10^{-12}	5.5×10^{-7}	1	
Blank 1[e]		1				
Blank 2[e]		0				
Capsule 1	0.055	53		0.68	8.0×10^{-7}	44.28
Capsule 2	0.061	54		0.32	1.7×10^{-6}	102.88
Capsule 3	0.064	52		0.51	1.1×10^{-6}	67.69
Capsule 4	0.061	56		0.45	1.2×10^{-7}	73.32
Capsule 5	0.055	48		0.37	1.5×10^{-6}	81.72
Capsule 6	0.056	50		0.40	1.4×10^{-6}	74.86

[a] Maximum luminescence elicited relative to that of E2 in the VM7Luc4E2 assay. [b] Concentration in which 50% of the maximum effect is observed. EC_{50} values are shown for the extracts of capsule coffee used in the VM7Luc4E2 assay. [c] Potency of capsule coffee relative to that of E2: $\boldsymbol{EEF} = \frac{EC50_{E2}}{EC50_{sample}}$. [d] Estradiol equivalent concentration, where $\boldsymbol{EEQ} = \boldsymbol{EEF} * \boldsymbol{Concentration}$. [e] Blank 1 and capsules 1–4 were used in one brand of capsule coffee brewing machine and blank 2 and capsules 5–6 were used in a second brand.

in the same machine, only two contained BPF. All capsule coffee samples contained at least one estrogenic chemical, and the number of total estrogenic chemicals ranged from 1 to 4, with capsule 2 containing BPF, BP, 4-NP and DBP. No estrogenic chemicals were detected in blank 1 and 2, indicating that there was no contamination from the brewing machine or other sources.

Table 2
Estrogenic chemical content of capsule coffee.

Samples	Estrogenic chemical content (ng/mL coffee)								
	BPA	BPF	BP	4-NP	DBP	DMTP	BPS	Caprolactam	DEHP
Blank 1	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Blank 2	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Capsule 1	*n.d.*	5.57	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Capsule 2	*n.d.*	5.12	143.27	7.39	9.27	9.27	*n.d.*	*n.d.*	*n.d.*
Capsule 3	2.42	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Capsule 4	2.16	*n.d.*	118.60	*n.d.*	9.35	9.35	*n.d.*	*n.d.*	*n.d.*
Capsule 5	*n.d.*	*n.d.*	30.51	4.37	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Capsule 6	*n.d.*	*n.d.*	149.40	*n.d.*	12.34	12.34	*n.d.*	*n.d.*	*n.d.*

Blank 1 and capsules 1–4 were used in one brand of capsule coffee brewing machine and blank 2 and capsules 5–6 were used in a second brand. 4-NP, 4-nonylphenol; BP, benzophenone; BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; DBP, dibutyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DMTP, dimethyl terephthalate; n.d., not detectable.

4. Discussion

This study sought to assess the estrogenic activity of capsule coffee using the VM7Luc4E2 estrogen receptor transcriptional activation assay. All capsule coffee samples were confirmed to have estrogenic activity, with potencies six to seven orders of magnitude weaker than that of E2. Five out of nine estrogenic chemicals tested were detected in the coffee samples and included BPA, BPF, BP, 4-NP and DBP.

The estrogenic activity of the capsule coffee samples is suspected to be partly due to the contamination with estrogenic chemicals. However, the extent to which these chemicals contribute to the capsule coffees' estrogenic activity is difficult to assess. BPA, BPF, BP, 4-NP and DBP have been confirmed to induce estrogenic activity in vitro or in vivo (Schafer et al., 1999; Rochester and Bolden, 2015; Moreman et al., 2017; Nakagomi et al., 2018; Nakagawa and Tayama, 2001; Hashimoto et al., 2003; In et al., 2015), but there is wide variability in the measurements of each chemical's estrogenic potential due to assay type and lab-specific methodology. For instance, the EEF for BPA was found to be ten times greater in a competitive binding assay compared to a luciferase reporter gene assay (Gutendorf and Westendorf, 2001). Additionally, since BPA (Rutishauser et al., 2004; Murk et al., 2002; Sun et al., 2008), BPF (Hashimoto et al., 2001) and 4-NP (Rutishauser et al., 2004; Sun et al., 2008) generally have higher estrogenic potentials than BP (Schreurs et al., 2005) and DBP (Murk et al., 2002), it is not feasible to aggregate and compare the total estrogenic chemical content to the estrogenic potential of capsule coffee, as one might do with total phytoestrogen or pesticide content. In a VM7Luc4E2 assay (Yang et al., 2014), BPA and 4-NP had EEF values ($EC50_{E2}/EC50_{EC}$) of 8.5×10^{-6} and 1.4×10^{-6}, respectively, which are weak in estrogenic potential and comparable to the EEF values of the capsule coffee obtained in the current study. In another study using the same assay technique (Peng et al., 2021), BPA and BPF possessed an E_{max} of slightly $<$ 60%RME2, which is also comparable to that of the coffee samples in our study. DMTP was not found to possess estrogenic potential in similar reporter gene assays, although did demonstrate estrogen receptor agonist activity in a cell proliferation assay (Peng et al., 2019). To our knowledge, the estrogenic potential of BP and DBP have not yet been tested using the VM7Luc4E2 assay. One pattern that did emerge from our analysis was that the number of estrogenic chemicals detected corresponded with estrogenic potential: capsule 2 possessed the highest estrogenic potential (EEQ = 102.88 ng/mL) and the largest number of estrogenic chemicals (4) with a relatively higher concentration of BPF, BP and 4-NP. Comparatively, capsules 1 and 3, each with one estrogenic chemical detected, had the lowest estrogenic potentials. In addition, blanks, in which no estrogenic chemicals were detected, did not exert estrogenic activity. This indicates a connection between the presence of estrogenic chemicals and the estrogenic potential of capsule coffee, although further investigations are required to fully understand the degree to which they are related and to predict the estrogenic potential based on the concentration of estrogenic chemicals.

As no estrogenic chemicals were detected in the blanks run through the coffee brewing machine, it is apparent that contamination occurs within the capsule. The capsules are typically composed of polypropylene which has been shown to leach estrogenic chemicals in food products (Yang et al., 2011; Rubio et al., 2019). High temperatures also significantly increase the rate of migration of estrogenic chemicals from plastic (Le et al., 2008; Cooper et al., 2011), due in part to the non-covalent bond between certain plasticizers to plastic (Rowdhwal and Chen, 2018). Di Bella et al. (Di Bella et al., 2014) demonstrated that plasticizers were evident in the coffee grounds that were removed from polypropylene and polystyrene capsules. Furthermore, there was a higher concentration of plasticizers in the coffee brewed from the capsule, suggesting that estrogenic chemicals migrate from the capsule just from making contact with coffee grounds and that brewing the capsule increases the amount of estrogenic chemicals migrated. Together with the results of the current study, there is evidence to indicate that consuming capsule coffee exposes one to estrogenic chemicals and that the capsule coffee is confirmed to have estrogenic activity in an in vitro cell model. Whether this exposure to estrogenic chemicals from capsule coffee is concerning to health, particularly to reproductive systems, is unknown and deserves investigation.

While the scope of this study was to evaluate the estrogenic activity of capsule coffee and relate it to the contamination of estrogenic chemicals, the contribution of other compounds purported to possess estrogenic activity that are inherent in coffee deserve acknowledgement. The isoflavones formononetin, daidzein, genistein and biochanin A comprise a significant portion of coffee flavonoids (Alves et al., 2010) and have been shown to possess a moderate degree of estrogenic activity in vitro (Yang et al., 2014; Takamura-Enya et al., 2003; Quirk et al., 2013). The strength of estrogenic activity of daidzein and genistein, the isoflavones abundant in soy products, has been reported to be comparable to (Yang et al., 2014) or weaker (Huang et al., 2007) than that of BPA. The phenolic acids chlorogenic acid, caffeic acid and ferulic acid, each present in coffee, induced reporter gene transcription in MCF-7 cells, indicating estrogenic activity (An et al., 2019). Notably, the VM7Luc4E2 assay has not been used to evaluate the estrogenic potential of flavonoids. However, there are some discrepancies regarding the estrogenic potential of some of these compounds. In human colon carcinoma Caco-2 cells, chlorogenic acid and caffeic acid failed to induce an effect on SULT1E1 expression (an in-activator of estrogen) (Isshiki et al., 2013). Differences in outcomes may be attributed to the dose administered or the type of in vitro model used. Caffeine, the stimulatory agent abundant in coffee, has been found to be either anti-estrogenic (Kiyama, 2019; Rosendahl et al., 2015) or have no estrogenic potential (Isshiki et al., 2013). It is thus likely that the flavonoid content contributed to the estrogenic potential of the capsule coffee samples, although the specific compounds and their degrees of contribution require further investigation.

While capsule coffee was determined to possess significant estrogenic activity, it is important to consider the intake of all food and beverages

with significant estrogenic potential when assessing exposure and potential risk to health. Omoruyi & Pohjanvirta (Omoruyi and Pohjanvirta, 2018) reported the estrogenic activities of 21 beer samples (range: 0–43.6 ng EEQ/mL) and indicated that the hops used in beer brewing were responsible for the estrogenicity. Specifically, the presence of 8-prenylnaringenin, a flavonoid with high estrogenic potential (Milligan et al., 1999), was purported to contribute to estrogenicity. Bottled water (Wagner and Oehlmann, 2009), fruits and vegetables (Schilirò et al., 2013) also possessed estrogenic activity, although with potencies far lower (bottled water: 75 ng EEQ/L, fruits and vegetables: 0.1 ng EEQ/g) than that of our capsule coffee. Soy-based food, which is rich in isoflavones, is highly estrogenic (tofu: 1544 ng EEQ/kg) (Behr et al., 2011) and frequently consumed in Asia, although is less common in Western countries. For reference, oral forms of estradiol for the treatment of climacteric symptoms/hormone replacement therapy are commonly administered in a dose of 2 mg daily (Ofri, 2001), which is approximately 100–1,000 times greater than the total estradiol equivalent load of a cup of capsule coffee. Compared to these other foods and beverages, the estrogenic potential of capsule coffee was relatively high, at least when considering per concentration. However, the total volume/weight of the food/beverage consumed must be considered; coffee is typically consumed in relatively small portions compared to water or beer. Thus, the overall exposure to estrogenic compounds other sources may equal or even exceed that of capsule coffee depending on the amount consumed. As this study tested the estrogenicity of capsule coffee in vitro it is not yet possible to make recommendations regarding intake as it relates to health outcomes, particularly concerning reproductive health. Analyses of toxicity in vivo and investigations on metabolism of estrogenic chemicals (both native to capsule coffee and through contamination) in humans will first be required. However, this study identified capsule coffee as a substance with significant estrogenic potential. Since coffee consumption is typically habitual and chronic, it is essential to continue to evaluate the risk of exposure to estrogenic compounds over a sufficient period of time.

This study had several strengths, most notably being the first evaluation of estrogenic activity of capsule coffee in vitro. It contributes to the safety assessment of consuming capsule coffee in regard to its estrogenic potential when there is currently scant evidence. Second, the UPLC-MS/MS analytical method for determining the estrogenic chemical content was validated for this study, thereby improving the validity of the results. However, as this was an exploratory study with the primary goal of determining whether capsule coffee exhibited estrogenic activity, the sample size was small and the findings are merely suggestive of a broad pattern. Second, it was not feasible to analyze all possible estrogenic chemicals so it is possible other estrogenic chemicals may be contributing the estrogenic potential. However, we targeted the major estrogenic chemicals that are likely to leach from plastic into coffee based on previous works (Di Bella et al., 2014; De Toni et al., 2017; Mohamed and Ammar, 2008; Guo et al., 2012).

In conclusion, capsule coffee samples were positive for estrogenic activity as determined by the VM7Luc4E2 assay. Contamination with estrogenic chemicals is evident in capsule coffee and is a possible driver of capsule coffee's estrogenic potential. As this study only assessed the estrogenic activities of capsule coffee, it will be important for future studies to additionally assess the estrogenic activities of the major estrogenic components in capsule coffee such as BPA, phthalates and isoflavones in order to determine the compounds contributing the most to its estrogenic potential. Future studies should also evaluate the relevance of these findings in animal and human intervention trials.

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CRediT authorship contribution statement

Junichi R. Sakaki: Formal analysis, Writing - original draft. **Melissa M. Melough:** Investigation, Writing - review & editing. **Cathy Z. Yang:** Validation, Data curation, Investigation, Methodology. **Anthony A. Provatas:** Validation, Data curation, Investigation, Methodology, Writing - review & editing. **Christopher Perkins:** Validation, Methodology. **Ock K. Chun:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2021.05.003.

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