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Survey of the *trans*-Resveratrol and *trans*-Piceid Content of Cocoa-Containing and Chocolate Products

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Dietary resveratrol (3,4′,5-trihydroxystilbene) has been implicated in the health benefits associated with grapes and red wine, more specifically with potential benefits for metabolic syndrome, energy use, and increased endurance. Levels of *trans*-resveratrol and its glucoside, *trans*-piceid, were determined in 19 top selling commercially available cocoa-containing and chocolate products from the U.S. market. Amounts of *trans*-resveratrol and *trans*-piceid were closely correlated with the amount of nonfat cocoa solids (NFCS) in the cocoa-containing products. Among these products, *trans*-resveratrol levels were highest in cocoa powders (1.85 ± 0.43 μg/g), followed by unsweetened baking chocolates (1.24 ± 0.22), semisweet chocolate baking chips (0.52 ± 0.14), dark chocolates (0.35 ± 0.08), milk chocolates (0.10 ± 0.05), and chocolate syrups (0.09 ± 0.02). These cocoa-containing and chocolate products have about 3−5 times more *trans*-piceid than *trans*-resveratrol. Levels of *trans*-piceid were highest in the cocoa powders (7.14 ± 0.80 μg/g), followed by unsweetened baking chocolates (4.04 ± 0.14), semisweet chocolate baking chips (2.01 ± 0.18), dark chocolates (1.82 ± 0.36), milk chocolates (0.44 ± 0.06), and chocolate syrups (0.35 ± 0.06). On an equal weight basis, cocoa powder had about half as much *trans*-resveratrol as the average California red wine. On a per serving basis, cocoa-containing and chocolate products had less *trans*-resveratrol than red wine and grape juice but more than roasted peanuts. Overall, these cocoa-containing and chocolate products rank second after red wines and grape juice in foods with the highest levels of total *trans*-resveratrol in the diet.

KEYWORDS: Resveratrol; piceid; stilbene; cocoa; cacao; chocolate; wine; peanut

INTRODUCTION

Recently, a number of studies have reported potential benefits of dietary resveratrol with respect to metabolic syndrome and food consumption (*1*−*3*). Resveratrol, (3,4′,5-trihydroxystilbene), is a member of the stilbene family and has been associated with the French Paradox (*4*). Its polyphenolic structure imparts antioxidant activity by forming a stable radical through resonance structures that prevents further oxidation, and there is evidence this antioxidant activity also occurs *in vivo* (*5*). Studies also have shown that consumption of resveratrol increases vasodilation (*6*), inhibits platelet aggregation and coagulation (*7*), as well as modifies eicosanoid synthesis and lipoprotein metabolism (*8*). Furthermore, some hypothesize that the structure of resveratrol suggests phytoestrogenic potential, which may further contribute to its potential cardioprotective effects (*9*).

Resveratrol can occur as the *cis* or *trans* stereoisomers, with the *trans* form being the dominant form and the focus of research (*1*). Resveratrol can also occur in a glucosylated form and is the building block for the class of compounds called viniferins (*10*). The 3-β-glucosylated form is called *trans*-piceid. In addition to *trans*-piceid, other related compounds include *trans*-piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene), *trans*-pterostilbene, and resveratrol polymers.

The parent *trans*-resveratrol has been detected in a wide variety of plant species, many of which are not edible (*11*). Primary dietary sources of resveratrol are grape and grape products (e.g., wine) and peanut products. Recently, *trans*-resveratrol and *trans*-piceid were detected in hops (*12*). Resveratrol concentrations vary widely even within a food source. In maturing grapes, resveratrol levels increase in response to plant stress, such as fungal and bacterial infection or UV radiation (*13*). Furthermore, levels of the compound in wine tend to be higher in grapes grown in cooler climates.

Cocoa (*Theobroma cacao* L.) is a rich source of antioxidant compounds, most notably the flavan-3-ol polyphenol class,

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Table 1. Top Selling U.S. Products in Six Chocolate and Cocoa-Containing Categories

product category	manufacturer/brand[a]
natural cocoa powder	Ghirardelli cocoa
	Hershey's cocoa
	Nestle Toll House cocoa
unsweetened chocolate	Baker's unsweetened baking chocolate
	Ghirardelli unsweetened chocolate baking bar
	Hershey's unsweetened chocolate baking bar
	Nestle unsweetened chocolate baking bar
dark chocolate	Hershey's special dark mildly sweet chocolate
	Lindt Excellence 70% cocoa
	Dove Promises dark chocolate
semisweet chocolate baking chips	Ghirardelli premium semi-sweet chocolate chips
	Hershey's semi-sweet chocolate chips
	Nestle Toll House semi-sweet chocolate morsels
milk chocolate	Hershey's milk chocolate
	Dove Promises milk chocolate
	Lindt Excellence extra creamy milk chocolate
chocolate syrup	Hershey's syrup
	Kroger syrup
	Nestle Nesquik syrup

[a] Manufacturers are listed alphabetically within product category. The product order in this table has no relationship to the order shown in **Table 2**.

Table 2. *trans*-Resveratrol and *trans*-Piceid Content of Leading Chocolate and Cocoa-Containing Products in the U.S. Marketplace

product code	nonfat cocoa solids (%)	fat (%)	*trans*-resveratrol (μg/g)	*trans*-piceid (μg/g)
		Cocoa Powder		
CP-1	87.3	12.4	2.27	8.17
CP-2	87.3	11.0	2.02	7.01
CP-3	72.3	21.7	1.25	6.23
average (±SD)	82.3 (7.1)	15.0 (4.7)	1.85 (0.43)	7.14 (0.80)
		Unsweetened Chocolate		
BC-1	49.5	51.6	1.39	3.81
BC-2	49.1	52.4	1.47	4.20
BC-3	46.6	53.1	1.19	4.08
BC-4	44.8	53.1	0.89	4.06
average (±SD)	47.5 (1.9)	52.6 (0.6)	1.24 (0.22)	4.04 (0.14)
		Semisweet Chocolate Chips		
SSC-1	18.6	29.0	0.58	2.19
SSC-2	15.2	29.8	0.33	1.76
SSC-3	17.0	27.8	0.66	2.09
average (±SD)	16.9 (1.4)	28.9 (0.8)	0.52 (0.14)	2.01 (0.18)
		Dark Chocolate		
DC-1	20.0	30.0	0.43	1.69
DC-2	20.7	33.4	0.38	1.47
DC-3	29.5	40.7	0.25	2.31
average (±SD)	23.4 (4.3)	34.7 (4.5)	0.35 (0.08)	1.82 (0.36)
		Milk Chocolate		
MC-1	6.4	31.4	0.09	0.43
MC-2	7.2	29.3	0.17	0.52
MC-3	4.9	37.0	0.05	0.37
average (±SD)	6.2 (1.0)	32.6 (3.2)	0.10 (0.05)	0.44 (0.06)
		Chocolate Syrup		
S-1	4.8	1.2	0.06	0.27
S-2	6.6	1.3	0.11	0.37
S-3	7.9	1.4	0.09	0.41
average (±SD)	5.7 (0.9)	1.3 (0.1)	0.09 (0.02)	0.35 (0.06)

which includes catechin, epicatechin, and procyanidins (*14*). Natural cocoa powder can contain over 2% of these compounds (*15*). In 2006, Counet et al. (*16*) reported the presence of *trans*-resveratrol and *trans*-piceid in single samples of cocoa liquor and dark chocolate, with 0.4−0.5 μg/g of resveratrol and 1.0−1.2 μg/g of *trans*-piceid. The present study was conducted to further characterize the levels of *trans*-resveratrol and *trans*-piceid in a variety of cocoa-containing and chocolate foods and to relate these foods to other significant dietary sources of resveratrol, such as red wine and peanut products.

MATERIALS AND METHODS

Sample Collection and Preparation. The top three selling products in each of the following product categories were obtained for analysis: natural cocoa powder, unsweetened baking chocolate, dark chocolate, semisweet chocolate baking chips, milk chocolate, and chocolate syrup (**Table 1**). Market share in the U.S. was determined from August 2004 sales data obtained from the Information Resources, Inc. (Chicago, IL). Because of a tie in the baking chocolate category, four products were collected. A total of 19 different products representing 7 major manufacturers were sampled. A minimum of 18 different production codes of each product were purchased at retail locations across the U.S. A statistical sampling design as described by Miller et al. (*15*) was used to make a composite sample of each product. All composite samples were stored at 0 °C prior to analysis. Samples were blinded for analysis.

Sample Analysis. Composite samples were analyzed in duplicate for nonfat cocoa solids (NFCS), total fat, *trans*-resveratrol, and *trans*-piceid. NFCS determination is a gravimetric method based on extraction of the sample with a variety of lipophilic solvents to eliminate the lipid content (*17*). The NFCS is a measure of the amount of brown cocoa particulates in a product and is a direct result of the product formulation. Chocolate liquor and cocoa powder contribute to the NFCS content of these products. Total fat was determined by Soxhlet extraction (*18*).

The high-performance liquid chromatography (HPLC) method previously described by Milbury (*19*) was modified to measure *trans*-resveratrol and *trans*-piceid. All samples were stored in darkness at 4 °C throughout the following operations with some samples ground, both before and after lipid removal with hexane, to improve the subsequent extraction. The cocoa powder samples did not require grinding.

For each of the samples, 5.00 g was delipidated with 40 mL of hexane for 3 h (3 times each). This delipidation time was held constant across all samples to cover their widely varying fat content. The samples were centrifuged between defatting steps and the hexane decanted. The remaining solid pellet was dried under vacuum at 35 °C to remove any residual hexane. The delipidated pellet was hydrolyzed for 8 h at 4 °C using 40 mL of a methanol/water/HCl solution (50:46.3:3.7, v/v/v). The solution was centrifuged, and the supernatant was collected. The hydrolysis process was repeated, and the two resulting supernatants were combined and treated with about 3.2 g of sodium bicarbonate to adjust the pH to 5. Organic solvents were removed by rotary evaporation in vacuo at 35 °C. The concentrated hydrolysate (approximately 25 mL) was extracted 2 times with 125 mL of ethyl acetate. Use of ethyl acetate as the extraction solvent eliminated significant quantities of water-soluble compounds, preventing unwanted precipitation and high viscosity of the final sample for the HPLC analysis. The ethyl acetate layer was evaporated to dryness and dissolved in 1.2 mL of methanol. Prior to injection, the samples were filtered and the eluent solution was used for HPLC analysis. All procedures were conducted in low light environments to minimize light-induced degradation. Extraction recoveries with ethyl acetate exceeded 99%.

All HPLC analyses were performed on a Hewlett-Packard 1090 instrument equipped with an autosampler, tertiary pump, column heater, and diode array detector. Separation was achieved using a 100 × 3.0 mm i.d., 3 μm YMC PackPro C-18 RP column, with a solvent gradient of acetonitrile/methanol (50:50, v/v) from 5 to 26% in 0.1% aqueous TFA over 40 min at a flow rate of 0.600 mL/min. The eluent was monitored at 310 nm. HP Chemstation version 10.03 was used for data collection and evaluation.

The quantity of *trans*-resveratrol and *trans*-piceid in the cocoa samples was calculated by comparison to absorption data from reference standards (*trans*-resveratrol from Sigma, R-5010; *trans*-piceid from Chromadex), injected at a concentration of 5 μg/mL and a volume of 4 μL. Each sample was analyzed in duplicate.

Statistical Analysis. Three additional samples from one composite sample in each food category were analyzed 2−3 times each to ensure method precision and determine the coefficient of variation (CV). The CV of the representative sample in each category was 2.1% for cocoa powder, 2.7% for baking chocolate, 4.1% for dark chocolate, 2.3% for semisweet chocolate baking chips, 3.1% for milk chocolate, and 6.2% for syrup. Linear correlation of NFCS with *trans*-resveratrol and *trans*-piceid, respectively, was analyzed using a force fit through the origin (NFCS = 0, variable = 0).

RESULTS AND DISCUSSION

Evaluation of the Method. The method described was evaluated using a number of analytical parameters to assess method suitability. An early observation was made that bound resveratrol was converted in an aqueous solution to free resveratrol. The result of these studies indicated that increased levels of resveratrol were not related to the hydrolysis of *trans*-piceid because *trans*-piceid was stable under these conditions. Furthermore, studies were conducted to ascertain the light-induced degradation of *trans*-resveratrol and the formation of *cis*-resveratrol using this method was not observed. Using UV detection at 310 nm, the lower limit of detection for *trans*-resveratrol and *trans*-piceid was found to be 200 and 170 pg, respectively, at twice the signal/noise ratio, and determined to be linear over the entire range of measured values. Additionally, the peaks of interest were not only determined by retention time but also by diode array detection (DAD)-derived UV spectra and spiking with reference standards. Finally, *trans*-resveratrol and *trans*-piceid standards (in triplicate) were subjected to the entire method, starting with the hydrolytic extraction step and ending with HPLC injection, with recoveries in excess of 75% for *trans*-resveratrol and 99% for *trans*-piceid. Subsequent sample analyses were corrected for the observed recovery. The use of HPLC with DAD as outlined in this paper is more practical, accommodating higher throughput of samples, compared to the liquid chromatography−mass spectrometry (LC/MS) method used previously by Counet et al. (*16*) to establish the presence of resveratrol and piceid in cocoa liquor and dark chocolate.

Levels of Resveratrol and Piceid in Products. Table 2 summarizes the NFCS, fat, *trans*-resveratrol, and *trans*-piceid for each of the products. Cocoa powders contained an average of 1.85 μg/g (±0.43; range of 1.25−2.27), the highest *trans*-resveratrol levels among the products in this study. Unsweetened baking chocolates contained the next highest level of *trans*-resveratrol (with an average of 1.24 μg/g ±0.22), followed by semisweet chocolate chips (0.52 ± 0.14 μg/g), dark chocolates (0.35 ± 0.08 μg/g), milk chocolates (0.10 ± 0.05 μg/g), and chocolate syrups (0.09 ± 0.02 μg/g).

Levels of *trans*-piceid in these chocolate and cocoa-containing products ranged from 3.2 to 5.2 times higher on average than the level of *trans*-resveratrol in the same product categories (**Table 2**). Cocoa powders had the highest average level of *trans*-piceid at 7.14 μg/g, followed by unsweetened baking chocolates (4.04 μg/g), semisweet chocolate chips (2.01 μg/g), dark chocolates (1.82 μg/g), milk chocolates (0.44 μg/g), and chocolate syrups (0.35 μg/g).

These data are the first to survey the occurrence of resveratrol and piceid in a variety of commercially available chocolates and cocoa-containing products. Concentrations in dark chocolates of *trans*-resveratrol fell close to the single previous report of at least 0.4 μg/g, while the average *trans*-piceid level of 1.8 μg/g was higher than the previously reported value of 1 μg/g *trans*-piceid (*16*). In general, the ranking from highest to lowest of these chocolate food categories is similar for both resveratrol and piceid, except that the levels of piceid are much higher on a gram of product basis. The same pattern has been observed in previous surveys of cocoa-containing products for measurements of oxygen radical absorbance capacity (ORAC), total

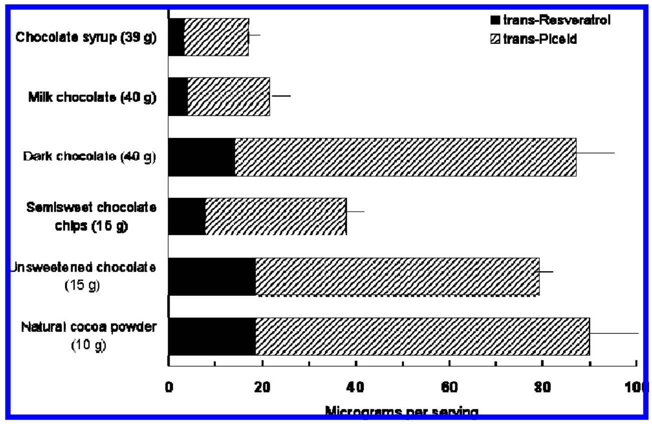

Figure 1. Average (±SD) of *trans*-resveratrol and *trans*-piceid content of leading chocolate and cocoa-containing products in the U.S. market.

polyphenols, and the flavan-3-ols (*14, 15*), with cocoa powders having the highest values, followed by unsweetened baking chocolates, dark chocolates, semisweet chocolate baking chips, milk chocolates, and syrups. This relationship suggests that both the stilbenes and the flavan-3-ols are associated with the brown cocoa particles in food formulation. Effects of food processing on levels of the stilbenes still need to be investigated.

Resveratrol and Piceid per Serving. When expressed on a per serving basis, cocoa powders and unsweetened baking chocolates provide similar amounts of *trans*-resveratrol, approximately 19 μg/serving (**Figure 1**). Dark chocolates have the next highest levels of total resveratrol per serving (average of 14.13 μg/serving). Semisweet chocolate chips have the next highest level of *trans*-resveratrol per serving (7.85 μg/serving), followed by milk chocolates (4.13 μg/serving) and then chocolate syrups (3.38 μg/serving). For *trans*-piceid, the highest levels were found in dark chocolate (72.9 μg/serving), followed by cocoa powders (71.4 μg/serving), unsweetened baking chocolates (60.6 μg/serving), semisweet chocolate chips (30.2 μg/serving), milk chocolates (17.6 μg/serving), and then syrup (13.6 μg/serving).

Other food sources reported to contain relatively high levels of *trans*-resveratrol and *trans*-piceid include grape products, including grape juice and red wines, and peanut products (**Table 3**). Red wines vary considerably in content, ranging anywhere from not detectable to as high as 1586 μg of *trans*-resveratrol (range of 0−1586 μg) and 3000 μg of *trans*-piceid per 150 mL serving (range of 0−3000 μg) in California and Brazilian red wines (*20, 21*). These values are about 14−45 times higher than the *trans*-resveratrol levels determined in the current study for servings of cocoa powder and baking chocolate. However, chocolate products appear to have similar amounts of *trans*-resveratrol per serving when compared to peanut products but higher levels of *trans*-piceid (*23*−*25*). This places cocoa powder, dark chocolate, and baking chocolate in a relatively high position for foods that contain resveratrol.

The percentage of NFCS in these products showed a strong linear correlation with both *trans*-resveratrol (**Figure 2**A; $R^2 = 0.9091$) and *trans*-piceid (**Figure 2**B; $R^2 = 0.99$). This correlation holds regardless of the basic composition of the food, namely, in powdered products (cocoa powder), fat-based products (unsweetened baking chocolates, dark chocolates, semisweet chocolate chips, and milk chocolates), or in water-based products (chocolate syrups). A similar linear correlation was observed previously for flavanols and ORAC in cocoa-containing products (*14, 15*). In contrast, the relationships were weak between % fat and *trans*-resveratrol ($R^2 = 0.1487$) and

Table 3. Comparison of Resveratrol Content of Red Wines, Grape Juice, Peanut Products, and Chocolate and Cocoa-Containing Products

food	serving size[a]	*trans*-resveratrol (μg/serving) (range)	*trans*-piceid (μg/serving) (range)	reference
red wines, California	150 mL	832 (80–1586)	ND	*20*
red wines, Brazilian	150 mL	297 (0–801)	1072 (ND–3000)	*21*
red grape juice	240 mL	120 (0–262)	811 (127–1762)	*22*
raw peanuts	28 g	5.5 (0.6–50.2)	not measured	*23*
roasted peanuts	28 g	1.5 (0.5–2.2)	not measured	*24*
peanut butter	32 g	10.3 (4.7–16.1)	not measured	*24*
peanut butter	32 g	17.0 (8.5–24.1)	4.3 (2.1–6.0)	*25*
cocoa powder	10 g	18.5 (12.5–22.7)	71.4 (62.3–81.7)	
baking chocolate	15 g	18.5 (17.8–22.0)	60.6 (57.2–63)	
dark chocolate	40 g	14.1 (10.0–17.2)	72.9 (67.6–92.4)	
semisweet chocolate chips	15 g	7.85 (4.9–9.9)	30.2 (32.8–26.4)	
milk chocolate	40 g	4.1 (2.0–6.8)	17.6 (14.8–20.8)	
chocolate syrup	39 g	3.4 (2.3–4.3)	13.6 (10.5–16.0)	

[a] Serving sizes are based on the U.S. Food and Drug Administration's Reference Amounts Customarily Consumed per Eating Occasion (21 Code of Federal Regulations 101.12). ND = not detected.

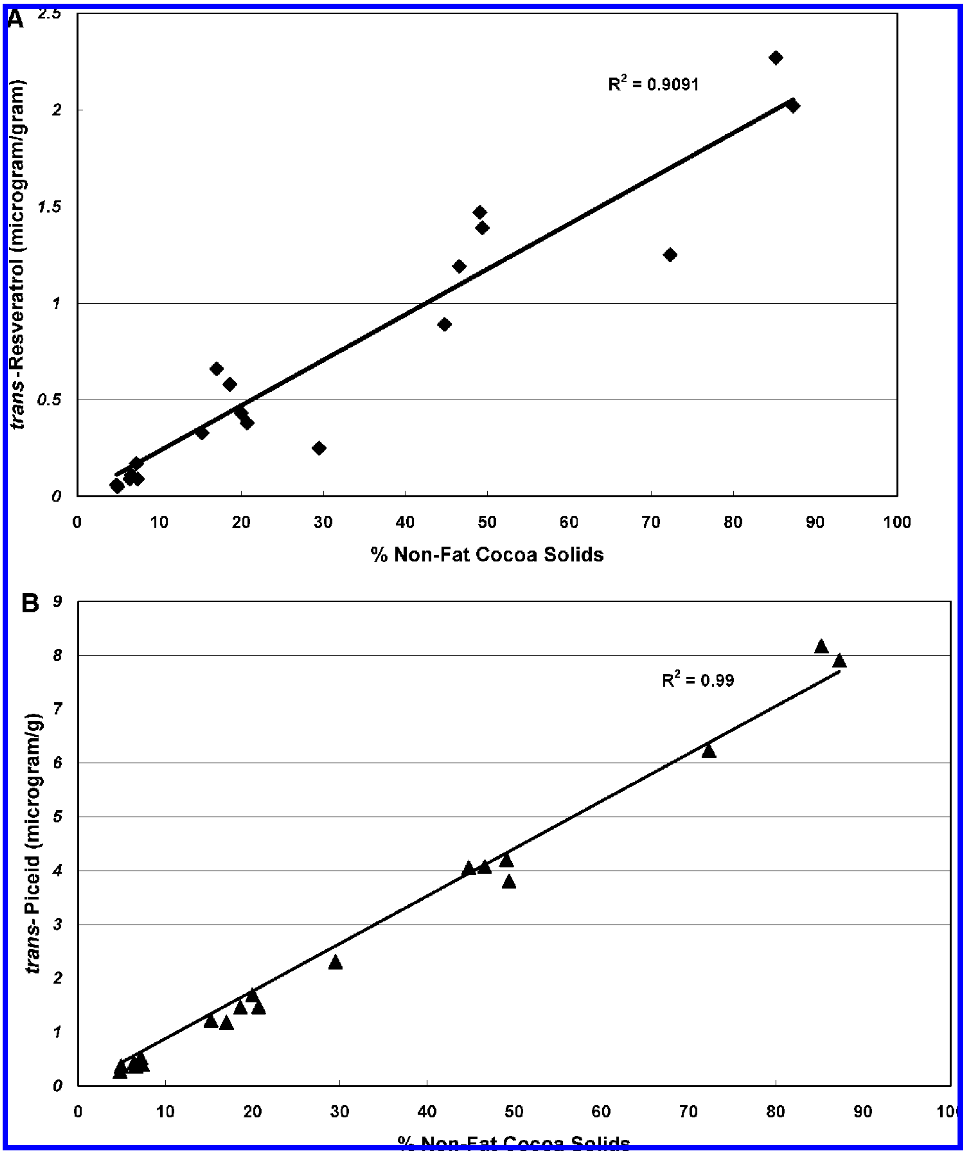

Figure 2. Relationship between NFCS and (A) *trans*-resveratrol or (B) *trans*-piceid as illustrated by the line describing the best linear fit of the data, with force fitting through the origin.

trans-piceid (R^2 = −0.1794) (data not shown). The data indicates that resveratrol and piceid are found in the nonfat portion of the cocoa bean.

Potential Physiological Significance. Evidence from mammalian systems is that *trans*-piceid may be absorbed as *trans*-resveratrol after being deglucosylated in the stomach (*26*). Previously observed *in vivo* physiological effects attributed to *trans*-resveratrol may be influenced by total stilbenes. If these stilbenes are equally absorbed and active, then cocoa- and chocolate-containing products are a significant dietary source of stilbenes. When the level of *trans*-piceid and *trans*-resveratrol are summed under the assumption that *trans*-piceid is deglycosylated and absorbed as *trans*-resveratrol, the level of these in cocoa-containing products is greatly increased. Finally, it is interesting to note that both *trans*-resveratrol (stilbenes) and the flavanols have been implicated in a number of common physiological effects, including vasodilation mediated by nitric oxide release (*27, 28*), inhibition of platelet aggregation (*29, 30*), and increased insulin sensitivity (*31, 32*). This suggests a potential common mode of action among these two polyphenolic classes, despite differences in their chemical structure biosynthesis, and the possibility of additive or synergistic effects when both are present in the diet.

ABBREVIATIONS USED

NFCS, nonfat cocoa solids; ORAC, oxygen radical absorbance capacity; % fat, the percentage of fat measured in the product or sample.

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