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Quantitative measurement of endogenous estrogen metabolites, risk-factors for development of breast cancer, in commercial milk products by LC–MS/MS☆

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ABSTRACT

Increased levels of estrogen metabolites (EM) are associated with cancers of the reproductive system. One potential dietary source of EM is milk. In this study, the absolute quantities of unconjugated (free) and unconjugated plus conjugated (total) EM were measured in a variety of commercial milks (whole, 2%, skim, and buttermilk). The results show that the milk products tested contain considerable levels of EM; however, the levels of unconjugated EM in skim milk were substantially lower than that observed in whole milk, 2% milk, and buttermilk. Whole milk contained the lowest overall levels of EM while buttermilk contained the highest. As anticipated, soy milk did not contain the mammalian EM measured using this method. The relatively high levels of catechol estrogens detected in milk products support the theory that milk consumption is a source of EM and their ingestion may have a dietary influence on cancer risk.

1. Introduction

Previously considered beneficial for their hormonal effects, estrogens are now, according to the past 20 years of epidemiological studies, considered to be risk factors of cancer of the breasts, ovaries, and prostate [1,2]. Estrogen metabolites (EM) are considered to be risk factors for multiple reasons: (1) increased exposure to EM leads to increased mitotic activity of endometrial cells, (2) increased exposure leads to an increase of DNA replication errors, and (3) somatic mutations often result in a malignant phenotype [1,3]. The greatest of the three risks is their ability to affect cellular proliferation. Animal studies have consistently shown that estrogens can promote mammary tumors in rodents. However, when the ovaries were removed and an antiestrogenic drug was administered, the tumors did not continue to grow [4]. Similar evidence that implicates EM in carcinogenesis is the fact that the incidences of EM-dependent diseases such as breast, ovarian, and prostatic cancer have been steadily growing in the past few years [5].

Though the relationship between EM and carcinogenesis has been extensively studied for decades, the data linking milk consumption with carcinogenesis are scarce. Nevertheless, the generated data have suggested that milk consumption correlates (directly or indirectly) with cancer development. For example, an experiment conducted by the Swedish Mammography Cohort showed a link between a woman's ovarian cancer risk and the quantity of milk consumed. Swedish women have one of the highest ovarian cancer rates in the world, which may be linked to the wide range of dairy products they consume [6]. This study showed that women who consumed ≥4 servings of total dairy products per day doubled their risk of serous ovarian cancer compared to women who consumed <2 servings per day. Similarly, women who consumed ≥2 glasses of milk per day had double the risk of serous ovarian cancer compared with women who never or seldom drank milk.

The correlation between milk consumption and the rate of breast and prostate cancers can also be seen within the post-World War II Japanese population. Previous to this war, Japan's milk consumption was virtually nonexistent. Today, however, Japan's milk consumption has increased 20-fold since World War II. This drastic change in dietary practice has led scientists to believe that the increased consumption of milk has led to higher incidences of breast and ovarian cancers. The average Japanese death rate resulting from breast cancer from 1948 to 1952 was 1374 people per year. From 1993 to 1997, this death rate increased to 7589 people per year [7]. The annual death rate due to prostate cancer in Japan has increased 13-fold since World War II and is increasing at the fastest rate in the world [8,9].

Animal-derived estrogens in the human diet are consumed primarily through milk and dairy products, constituting 60–70% of the total estrogen intake. Modern genetically improved dairy cows, such as Holsteins, are usually fed a combination of grass and concentrates (grain/protein mixes and various by-products), allowing

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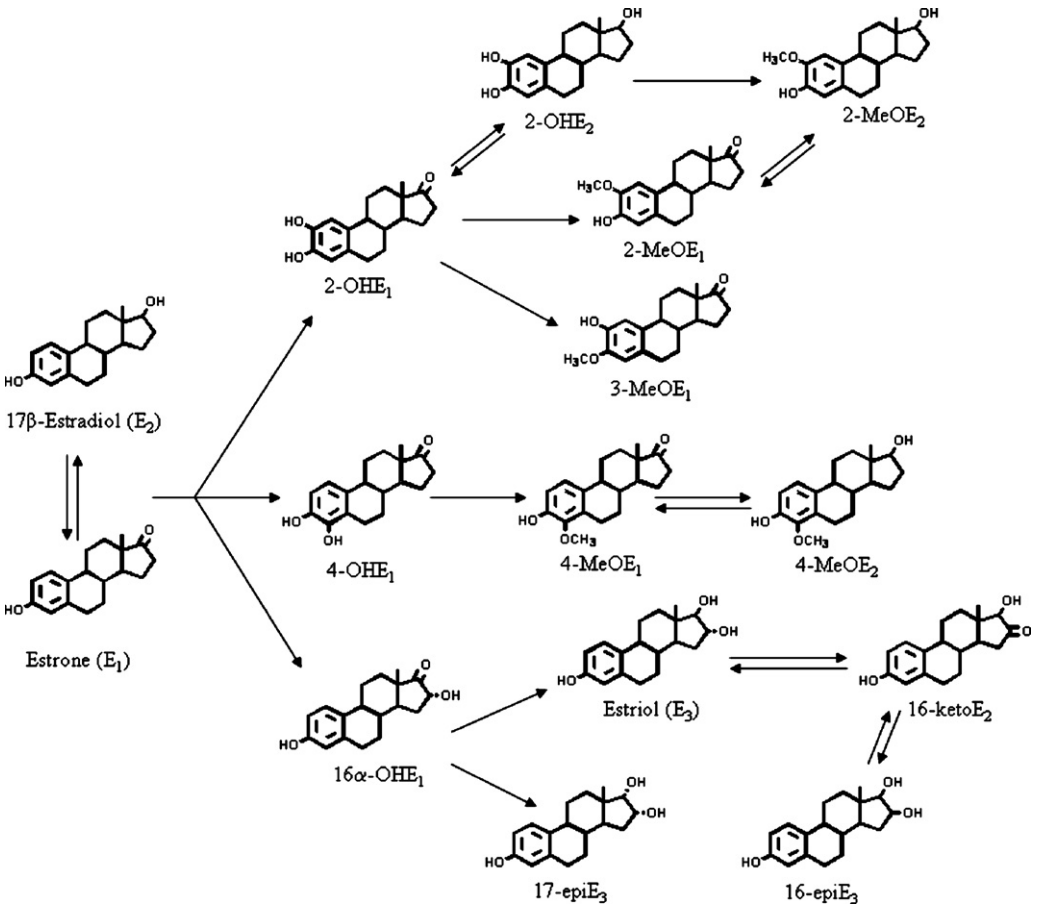


Fig. 1. Fifteen endogenous estrogen metabolites measured in various milk products in this study arranged within their metabolic pathways.

them to lactate during the latter half of pregnancy as late as 220 days into gestation [8]. Modern dairy practices have increased human EM intake since cows are milked far into their pregnancy when EM levels are markedly elevated. Japanese researchers have shown that milk from a cow in the late stage of pregnancy contains up to 33 times as much estrone sulfate than milk from a non-pregnant cow [10]. Researchers at Pennsylvania State University showed that estrone (E_1) concentrations averaged 0.6, 7.9, and 27.1 pg/mL through trimesters 1, 2, and 3, respectively, while estradiol (E_2) levels averaged 0.3, 0.9, and 5.0 pg/mL [11]. Researchers in the Netherlands discovered that the E_1 and βE_2 concentrations in milk increased over 16-fold and 2.5-fold between the first and third trimester of pregnancy, respectively [12]. These findings are alarming, considering that most commercial milk products originate from cows in the late stages of pregnancy. Increased EM levels appear to be conterminous with the development of the modern dairy industry, as dairy cows are often artificially inseminated three months after calving instead of being allowed to mate naturally. Unfortunately, pasteurization processes are incapable of inactivating estrogenic hormones before the milk is sold to customers [13].

The need for further research in regards to milk consumption and increased cancer risk has been well recognized [3,6,10,13–15]. Despite the significant toxicological consequences very little research has been conducted in an effort to quantify the levels of EM in various milk products. The similarity in structure of the EM is largely responsible for this problem, as they are difficult to separate and quantify in a single experiment. In this study, the levels of eleven commonly found EM in human circulation (Fig. 1) were measured in various types of commercially available bovine milk products. The results show that the variety of milk products tested differ in the level of EM they contain and provide a basis by which to correlate exact values of EM in various milk products to cancer risk.

2. Experimental

2.1. Reagents and materials

Standard compounds corresponding to eleven estrogen and estrogen metabolites (EM) analyzed in this study, estriol (E_3), 16-epiestriol (16-epiE_3), 2-methoxyestrone (2-MeOE_1), 4-methoxyestrone (4-MeOE_1), 2-methoxyestradiol (2-MeOE_2), estrone (E_1), 4-methoxyestradiol (4-MeOE_2), 17β-estradiol (E_2), 2-hydroxyestrone (2-OHE_1), 2-hydroxyestradiol (2-OHE_2), 4-hydroxyestrone (4-OHE_1), were obtained from Steraloids, Inc. (Newport, RI). The five deuterium-labeled estrogens and estrogen metabolites (*d*-EM), estradiol-2,4,16,16-d_4 (d_4-E_2), estriol-2,4,17-d_3 (d_3-E_3), 2-hydroxyestradiol-1,4,16,16,17-d_5 (d_5-2-OHE_2), and 2-methoxyestradiol-1,4,16,16,17-d_5 (d_5-2-MeOE_2) that were used as internal standards were purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). A fifth *d*-EM, 16-epiestriol-2,4,16-d_3 (d_3-16-epiE_3), was obtained from Medical Isotopes, Inc. (Pelham, NH). The spectra for d_4-E_2 and d_5-2-OHE_2 as provided in Fig. 2 show no evidence of observable exchange loss. None of the deuterated internal standards used in this study showed any appreciable exchange loss. All EM and *d*-EM were used without further purification and have reported chemical and isotopic purity ≥98%. Dichloromethane, methanol, and formic acid were obtained from EM Science (Gibbstown, NJ). Glacial acetic

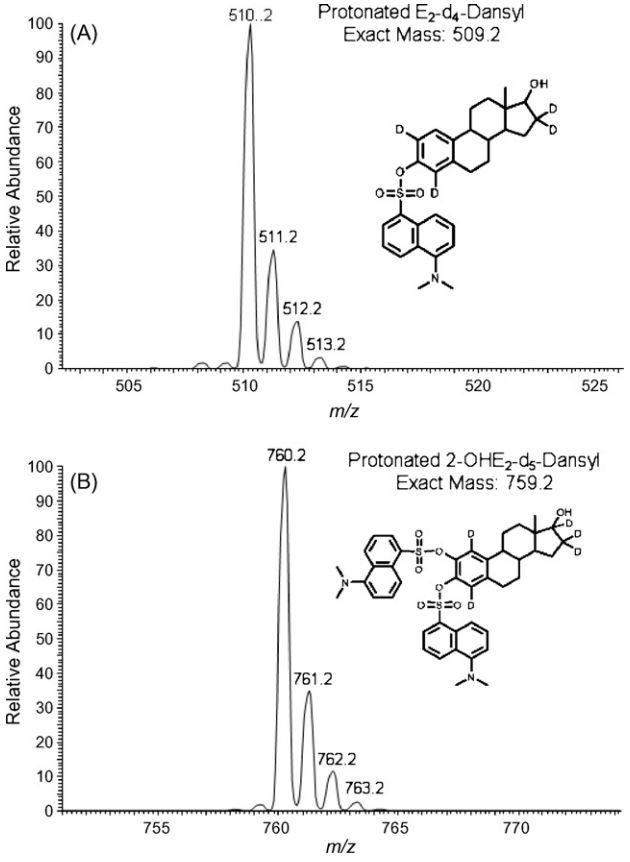


Fig. 2. Spectra showing the mass analysis of the dansylated forms of the deuterated internal standards d_4-E_2 and d_5-2-OHE_2 used in this study.

acid, sodium bicarbonate, and L-ascorbic acid were purchased from J.T. Baker (Phillipsburg, NJ). Sodium hydroxide and sodium acetate were purchased from Fisher Scientific (Fair Lawn, NJ). β-Glucuronidase/sulfatase (*Helix pomatia*, Type HP-2) was obtained from Sigma Chemical Co. (St. Louis, MO). Dansyl chloride and acetone were purchased from Aldrich Chemical Co. (Milwaukee, WI). All chemicals and solvents used in this study were HPLC or reagent grade.

2.2. Milk samples

Five milk samples were collected for this study: whole milk, 2% milk, skim milk, buttermilk, and soymilk. All Bloom™ milks (Bloom, Salisbury, NC) were purchased at a local grocery store (Bloom, Frederick, MD, U.S.A.). The five samples were analyzed in triplicate. Milk was aliquoted and stored at −40 °C until analyzed.

2.3. Preparation of stock and working standard solutions

Stock solutions of EM and *d*-EM were prepared at 80 μg/mL by dissolving 2 mg of each EM powder in 25 mL of methanol containing 0.1% (w/v) L-ascorbic acid. Time-dependent degradation of the EM and *d*-EM standards within the stock solutions was monitored by measuring the absolute peak height of each EM using capillary liquid chromatography–tandem mass spectrometry (LC–MS/MS), with no degradation observed for solutions stored at least two months at −20 °C. Working standard solutions of EM and *d*-EM at 8 ng/mL were prepared by diluting the stock solutions with methanol containing 0.1% (w/v) L-ascorbic acid.

Table 1
Accuracy (%)[a] of quality control samples for estrogen measurements.

	8 pg/mL	40 pg/mL	160 pg/mL
E_3	103.1	109.6	100.4
16-epiE_3	101.7	104.4	103.7
2-MeOE_1	103.5	98.0	102.0
4-MeOE_1	96.1	98.0	100.9
2-MeOE_2	97.8	99.0	101.0
E_1	101.5	101.9	100.5
4-MeOE_2	91.2	97.3	98.2
E_2	97.2	99.8	98.9
2-OHE_1	103.4	95.3	97.9
2-OHE_2	106.7	93.7	98.3
4-OHE_1	99.1	101.7	96.8

[a] Accuracy was measured as the percent matching of calculated amount to known amount of EM in control samples.

2.4. Calibration standards

Milk fortified with 0.1% (w/v) L-ascorbic acid and having no detectable levels of EM was employed for preparation of calibration standards and quality control (QC) samples. Calibration standards were prepared in milk by adding 20 μL of the *d*-EM working internal standard solution (0.16 ng *d*-EM) to various volumes of EM working standard solution, which typically contained 0.002–2 ng of each EM. Each calibration standard was assayed in duplicate. QC samples were prepared containing 8, 40, and 160 pg/mL (26.5–29.6, 132.4–148.0, 529.5–592.2 fmol/mL) of each EM. The accuracy (measured as the percent matching of calculated amount to known amount of EM in control samples) and precision (measured as the percent relative standard deviations) of the QC samples are provided as Tables 1 and 2. Calibration curves generated for each EM are provided in Fig. 3. These calibration curves were linear over a 10^3-fold range (0.2–200 pg on column) with R^2 greater than 0.996.

2.5. Sample preparation procedure

The sample preparation procedures [16,17] were designed to specifically target the following: (1) biologically active EM, which include unconjugated parent EM and their phase I metabolites as well as *O*-methylated catechol EM (i.e. 2-methoxyestrone, 4-methoxyestrone, and 2-methoxyestradiol), and (2) total (conjugated + unconjugated) estrogens after sulfatase and glucuronidase hydrolysis, which include biologically active EM plus their sulfate and/or glucuronide conjugates. For measuring total milk EM, 20 μL of the *d*-EM working internal standard solution (0.16 ng *d*-EM) was added to a 2 mL aliquot of milk, followed by addition of 7 mL of methanol. After 30 min of inverse extraction, the sample was centrifuged at 2500 × *g* for 30 min. The supernatant was transferred to a clean screw-capped glass tube, and the methanol portion

Table 2
Precision (%)[a] of quality control samples for estrogen measurements.

	8 pg/mL	40 pg/mL	160 pg/mL
E_3	0.87	0.99	0.22
16-epiE_3	8.3	0.40	2.5
2-MeOE_1	5.6	2.7	3.8
4-MeOE_1	4.0	3.5	2.8
2-MeOE_2	10.8	5.2	5.6
E_1	7.1	5.9	4.3
4-MeOE_2	1.7	2.0	2.2
E_2	6.4	3.3	2.9
2-OHE_1	5.3	5.0	3.2
2-OHE_2	4.9	5.4	3.5
4-OHE_1	8.8	6.0	8.7

[a] Precisions were measured as the percent relative standard deviations.

was removed using a stream of N_2 gas and by heating at 60 °C (Reacti-Vap III™, Pierce, Rockford, IL). Freshly prepared enzymatic hydrolysis buffer (0.5 mL) containing 5 mg of L-ascorbic acid, 15 μL of β-glucuronidase/sulfatase and 1.5 mL of 0.15 M sodium acetate buffer (pH 4.6) was prepared as previously described [16,17]. After sulfatase and glucuronidase hydrolysis by incubation for 20 h at 37 °C, 8 mL of dichloromethane was added and the samples underwent slow inverse extraction at 8 rpm (RKVSD™, ATR, Inc., Laurel, MD) for 30 min. After extraction, the organic solvent portion was transferred into a clean glass tube and evaporated to dryness at 60 °C under N_2 gas (Reacti-Vap III™, Pierce, Rockford, IL). To the dried sample, 100 μL of 0.1 M sodium bicarbonate buffer (pH at 9.0) and 100 μL of dansyl chloride solution (1 mg/mL in acetone) were added. The sample was vortexed and heated at 60 °C (Reacti-Therm III™ Heating Module, Pierce, Rockford, IL) for 5 min to produce the EM and *d*-EM dansyl derivatives (EM-Dansyl and *d*-EM-Dansyl, respectively). Calibration standards were hydrolyzed, extracted, and derivatized following the same procedure. After derivatization,

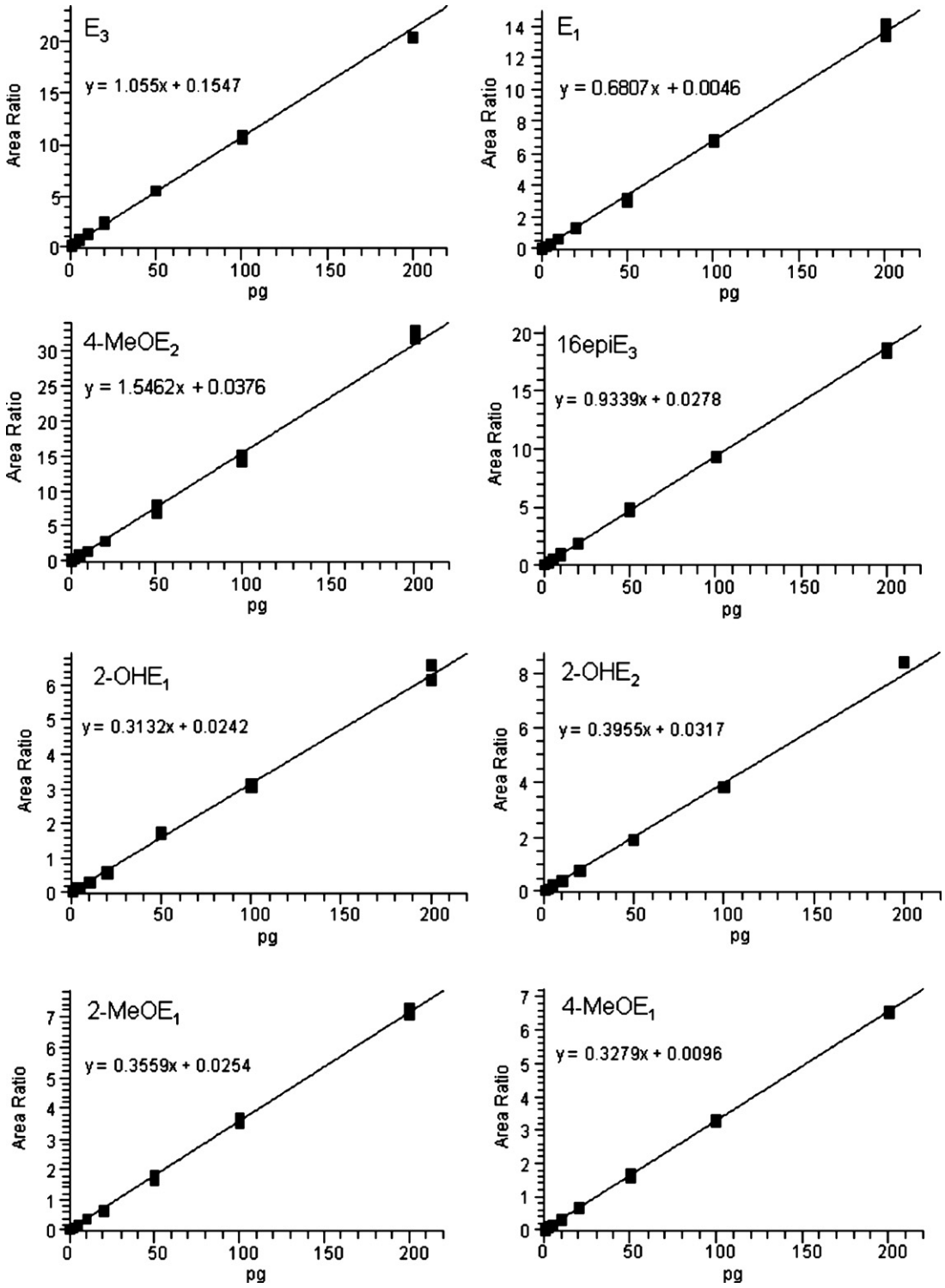


Fig. 3. Calibration curves, and their related equations, for each estrogen metabolite measured in the various milk products tested.

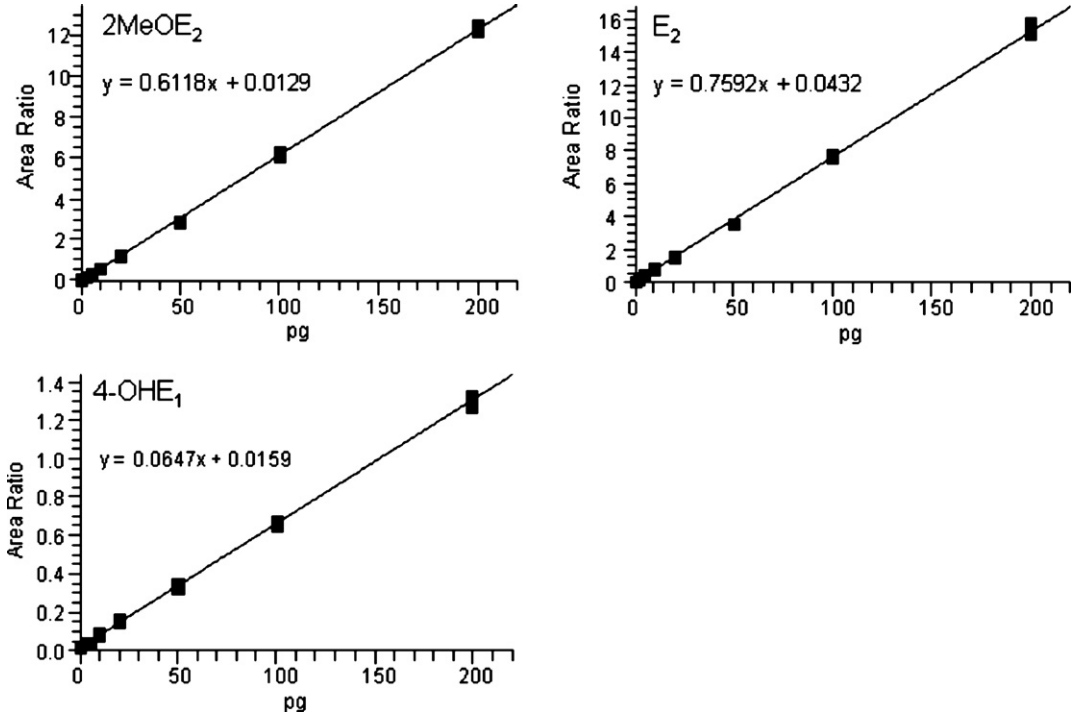


Fig. 3. (*Continued*).

all samples were analyzed by LC–MS/MS. For the measurement of total (unconjugated plus conjugated) EM, identical sample preparation was used with the omission of the β-glucuronidase/sulfatase hydrolysis step.

2.6. Capillary liquid chromatography–tandem mass spectrometry analysis (LC–MS/MS)

Quantitative analysis of EM was performed using an Agilent 1200 series nanoflow LC system (Agilent Technologies, Palo Alto, CA) coupled to a TSQ™ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA). The LC separation was conducted using a 150 mm long × 300 μm i.d. column packed with 4 μm Synergi Hydro-RP particles (Phenomenex, Torrance, CA), maintained at 40 °C. A total of 8 μL of each sample was injected onto the column. The mobile phases consisted of methanol as solvent A and 0.1% (v/v) formic acid in water as solvent B. A linear gradient from 72 to 85% solvent B in 75 min, at a flow-rate of 4 μL/min, was employed for separation of EM and SI–EM. The MS conditions were as follows source: ESI; ion polarity: positive; spray voltage: 3500 V; sheath and auxiliary gas: nitrogen; sheath gas pressure: 7 arbitrary units; ion transfer capillary temperature, 270 °C; scan type: selected reaction monitoring (SRM); collision gas: argon; collision gas pressure: 1.5 mTorr; scan width: 0.7 u; scan time: 0.50 s; Q1 peak width: 0.70 u full-width half-maximum (FWHM); Q3 peak width: 0.70 u FWHM. The optimized SRM conditions for the protonated molecules [M+H$^+$] of EM-Dansyl and *d*-EM-Dansyl were similar to those previously described [16,17]. Briefly, E_1 *m/z* 504→171 collision energy: 32 eV; E_2 *m/z* 506→171 collision energy: 35 eV; E_3, 16-epiE_3 *m/z* 522→171 collision energy: 33 eV; 2-$MeOE_1$ and 4-$MeOE_1$ *m/z* 534→171 collision energy: 37 eV; 2-$MeOE_2$ and 4-$MeOE_2$ *m/z* 536→171 collision energy: 36 eV; 2-OHE_1 and 4-OHE_1 *m/z* 753→170 collision energy: 44 eV; 2-OHE_2 *m/z* 755→170 collision energy: 43 eV; d_4-E_2 *m/z* 510→171 collision energy: 35 eV; d_3-E_3 and d_3-16-epiE_3 *m/z* 525→171 collision energy: 33 eV; d_5-2-$MeOE_2$ *m/z* 541→171 collision energy: 36 eV; d_5-2-OHE_2 *m/z* 760→170 collision energy: 43 eV.

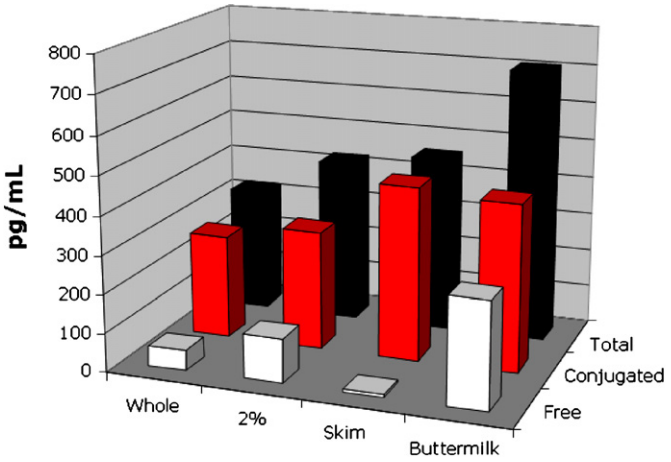
2.7. Quantitation of estrogen metabolites (EM)

Quantitation of milk EM was carried out using Xcalibur™ Quan Browser (ThermoFinnigan). Calibration curves for each EM were constructed by plotting EM-Dansyl/*d*-EM-Dansyl peak area ratios obtained from calibration standards versus amounts of EM and fitting these data using linear regression with 1/*X* weighting. The amount of EM in milk samples was interpolated using this linear function. To ensure the precision of the quantitative analyses, only deuterium-labeled estrogen standards that did not suffer exchange loss were employed in this study. Based on their similarity of structures and retention times, d_4-E_2 was used as the internal standard for E_2 and E_1; d_3-E_3 for E_3; d_3-16-epiE_3 for 16-epiE_3; d_5-2-$MeOE_2$ for 2-$MeOE_2$, 4-$MeOE_2$, 2-$MeOE_1$, and 4-$MeOE_1$; d_5-2-OHE_2 for 2-OHE_2, 2-OHE_1, and 4-OHE_1.

3. Results and discussion

3.1. Overall levels of estrogen metabolites in milk products

The sum of the absolute levels of free (i.e. unconjugated), conjugated (i.e. total minus free), and total EM measured in the four commercially available milk products are presented in Fig. 4. The amounts of conjugated EM were determined by subtracting the levels determined for the unconjugated EM from the total levels of EM. Buttermilk, whole milk, and 2% milk all showed considerable levels of EM in the unconjugated, biologically active form. Skim milk, however, showed surprisingly lower levels of EM (7.21 pg/mL in total). The overall range of unconjugated EM found in the milk products tested ranged from 7.21 to 273.72 pg/mL (Fig. 4). Whole milk had a total unconjugated EM level of 51.64 pg/mL. Buttermilk contained the highest total amount of unconjugated EM (273.72 pg/mL) amongst the milk products analyzed. The range of total EM detected in the four milk products tested was between 322.68 and 707.55 pg/mL (Fig. 4). The amounts of total EM in each milk product are significantly higher than that of unconjugated EM showing that most of the EM exists in conjugated forms (i.e. sul-



	Whole	2%	Skim	Buttermilk
Free	51.64	116.17	7.21	273.72
Conjugated	271.04	308.92	450.45	433.83
Total	322.68	425.09	457.66	707.55

Fig. 4. Bar chart showing the sum of the absolute levels of free (i.e. unconjugated), conjugated (i.e. total minus free), and total (unconjugated + conjugated) estrogen metabolites (EM) measured in commercially available whole, 2%, skim, and buttermilk. All values are given in pg/mL.

fated, glucoronidated, etc.). The discrepancy between unconjugated and conjugated forms was greatest for skim milk, where the results suggest that over 98% of the EM in this liquid is conjugated.

Not surprisingly, we were not able to detect any appreciable amounts of the EM measured in this study in soy milk. This observation is due to the fact that the structures of the estrogenic compounds in soy are not the same as those found in milk obtained from mammalian sources. Therefore, the method used in this study would not detect these soy-based compounds.

3.2. Levels of specific estrogen metabolites in milk products

The absolute levels and proportion of each of the unconjugated EM measured in whole, 2%, skim, and buttermilk are given in Fig. 5 and also supplied within supplemental Tables 1 and 2. In instances where the measurement is designated ND (not determined) the level of the EM was below the limit of quantitation of the assay, which is approximately 0.2 pg/mL [16,17]. As an example, the measurement of E_2 and 2-OHE_2 in all of the milk products analyzed in this study are shown in Figs. 6 and 7, respectively. While E_2 showed peaks with good signal-to-noise ratios in all of the samples analyzed, there was considerable variation in the peak size of 2-OHE_2 in the various milk products. For example, no peak could be measured for the free-form of this metabolite in buttermilk, while all milk products showed a large peak for the total amount of 2-OHE_2. The absolute levels of all of the EM detected in this study are provided in supplemental Tables 1 and 2, with the standard deviations of each metabolite measured in triplicate. Nearly 70% (36.05 pg/mL) of the EM content in whole milk was found to be 4-OHE_1, a highly reactive catechol estrogen. Likewise, 2% milk showed a large amount of EM (116.18 pg/mL), with 4-OHE_2 constituting nearly 70% (80.78 pg/mL) of this total. While buttermilk contained the highest levels of free and total EM of the milks tested, it did not contain any detectable levels of 4-OHE_1. Surprisingly, 2-OHE_1, another highly reactive catechol estrogen, constitutes over 95% (262.30 pg/mL) of the EM content in buttermilk. Clearly, the concentrations of E_1, 2-OHE_1, and 4-OHE_1 are disproportionally high compared to other EM, the latter two being of the most interest, as these are highly reactive catechol EM.

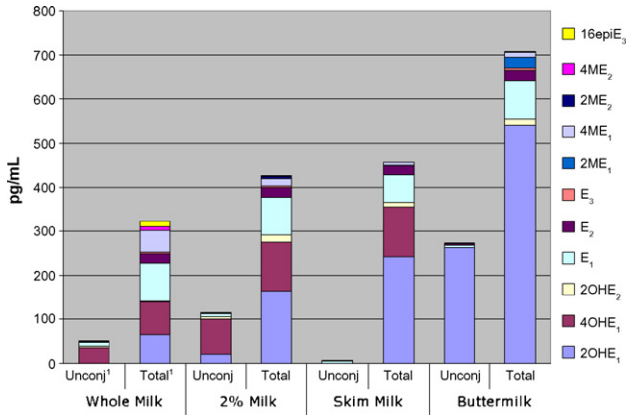


Fig. 5. Bar chart showing the absolute levels and proportion of each of the unconjugated (free) and unconjugated + conjugated (total) estrogen metabolite (EM) measured in whole milk, 2% milk, skim milk, and buttermilk. All values are given as pg/mL.

In skim milk, considerable levels of total E_1 (65.0 pg/mL), 2-OHE_1 (242.34 pg/mL), and 4-OHE_1 (111.38 pg/mL) were detected (supplemental Tables 1 and 2). Similar to skim milk, 2% milk also displayed elevated levels of total E_1 (84.56 pg/mL) 2-OHE_1 (164.35 pg/mL) and 4-OHE_1 (111.96 pg/mL). The obvious difference between these two types of milks is that 2% milk displayed elevated levels of these catechol estrogens in the free-form, whereas skim milk did not. In particular, 2% milk displayed significant levels of unconjugated 4-OHE_1 (80.78 pg/mL), whereas this highly reactive catechol EM was undetectable in skim milk. The fact that skim milk contained very low levels of unconjugated EM may be due to the higher aqueous composition of this milk product compared to the others that were tested, or the specific processing method used in the production of skim milk.

The total levels of the three catechol EM, E_1, 2-OHE_1, and 4-OHE_1, were also noteworthy in whole milk. While the levels of total E_1 in whole, 2%, and skim milk were similar (i.e. ranging from 65.00 to 85.46 pg/mL) whole milk contained only two thirds of the amount of total 4-OHE_1 as 2% and skim milk, and between 2.5 and 4-fold less 2-OHE_1 compared to 2% and skim milk.

Buttermilk contained the highest concentrations of total E_3, 2-$MeOE_1$, E_1, E_2, and 2-OHE_1. Though all EM concentrations in this

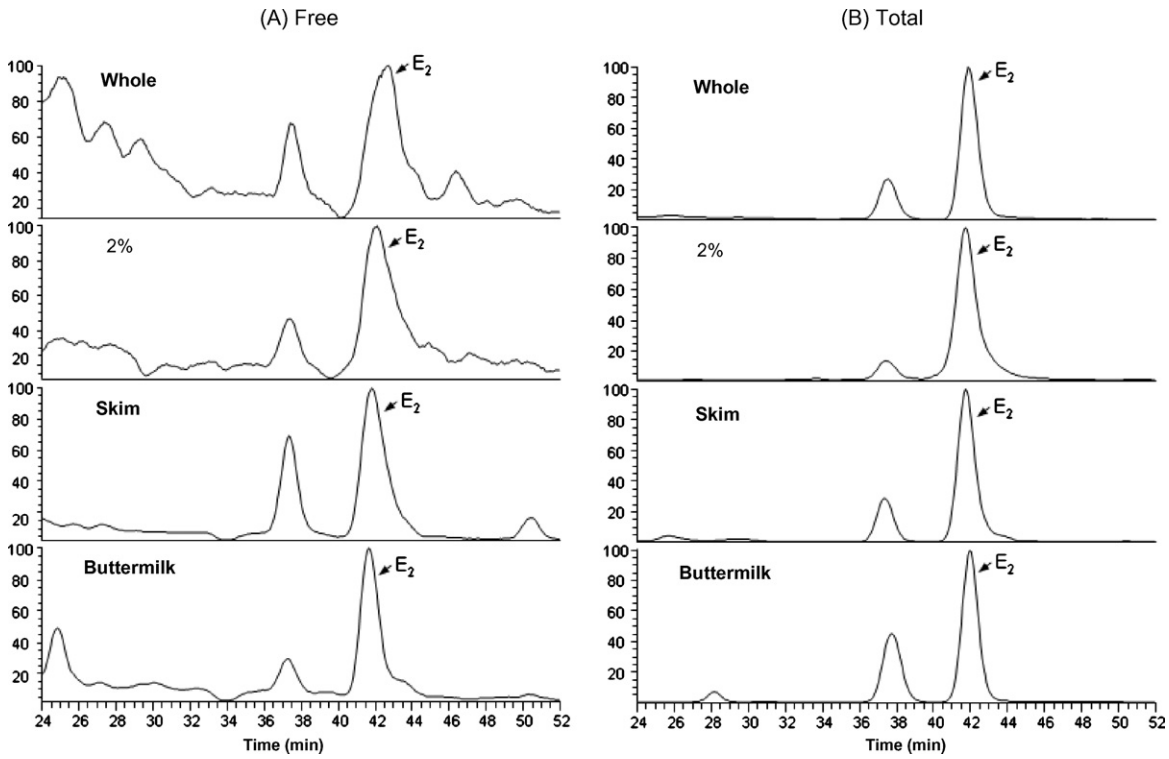


Fig. 6. High performance liquid chromatography–tandem mass spectrometry selected reaction monitoring (SRM) chromatographic profiles of (A) free (unconjugated) and (B) total (unconjugated + conjugated) E_2 in whole, 2%, skim, and buttermilk.

type of milk are quite high compared to the other types of milk tested, the main metabolite of concern is 2-OHE_1, a highly reactive catechol estrogen that buttermilk seems to contain in unusually high quantities in comparison with the other milks. As indicated by Fig. 7, 2-OHE_1 is the main estrogen constituent in buttermilk (total and free-forms). The only other milk product that contained 2-OHE_1 in its free-form was 2% milk (20.26 pg/mL); however, the level of this catechol EM was 13-fold greater in buttermilk.

Admittedly, the levels of conjugated EM measured in this study (i.e. approximately 450 ng/L) are low compared to those adminis-

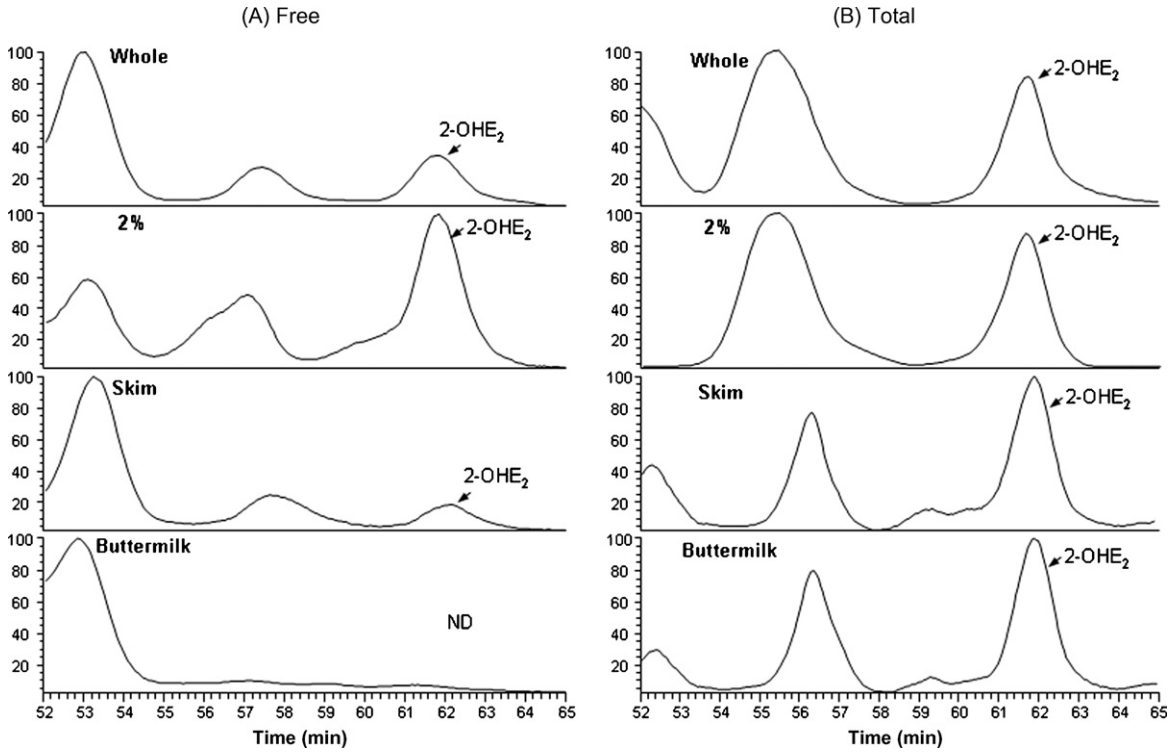


Fig. 7. High performance liquid chromatography–tandem mass spectrometry selected reaction monitoring (SRM) chromatographic profiles of (A) free (unconjugated) and (B) total (unconjugated + conjugated) 2-OHE_2 in whole, 2%, skim, and buttermilk.

Table 3
Comparison of estrogen metabolite levels in milk products and human serum.

	Milk (pg/mL)	Serum (pg/mL)
Free (unconjugated)		
E_1	3.5–10.2	3.0–133.0
E_2	1.0–2.4	2.0–266.0
Total		
E_1	65.0–87.9	192.6–1270.2
E_2	20.4–25.2	13.2–218.5

tered during hormone therapy. The level in a liter of skim milk, for example, is approximately 667 times lower than the conjugated equine estrogens in low-dose Premarin (300 μg) and 1389 times lower than standard dose Premarin (625 μg), which is associated with breast cancer incidence in post-menopausal women after long-term exposure [18,19]. Although the estrogen dose received via milk ingestion is much less compared to typical hormone therapies, the discovery of relatively high concentrations of catechol estrogens in all four types of milk is a very important result of our research, as we initially set out to develop a highly sensitive and specific LC–MS/MS method for measuring EM in various milk products, with special interest in the catechol estrogens. This highly sensitive and specific LC–MS/MS method has been successful in quantifying EM in three commonly consumed types of commercial milks (whole, 2%, and skim milk). Most important to note, this method has successfully quantified three highly reactive catechol estrogens in a *very complex* sample matrix. The effects of chronic exposure to catechol estrogens, which can be readily converted to mutagenic estrogen quinones, are presently unknown. The fact that they are present in all the commercial milks analyzed, estrogen intake through these products requires further investigation, considering the potentially harmful effects of these EM. This new methodology for potentially quantitating eleven EM in various commercial milks could be utilized in order to monitor the estrogen intake, specifically the catechol estrogens. Considering this is a relatively new field of research, it is necessary to further investigate the possible links milk consumption has to carcinogenesis, as milk is a very commonly consumed product.

A comparison of the levels of E_1, and E_2 found in the various milk products tested in this study and that found in human circulation [20,21] is provided in Table 3. The ranges provided cover the levels found in all of the milk products tested and the complete range found in serum from men, as well as pre- and post-menopausal women. While the levels these metabolites in milk are definitely within the low range of that found in human serum, the effect of long-term (i.e. possibly decades) ingestion at these levels is presently unknown. In addition, milk products are a major part of many infant's diets and therefore the proportionate dose compared to body mass is much greater than for an adult that may be on hormone replacement therapy.

The observation that the total levels of conjugated EM are much greater than that found in the unconjugated form is significant. A previous study comparing the administration of unconjugated E_1 and E_2 to that of estrogen sulfate and conjugated equine EM showed a more protracted influx of EM from the intestines to the plasma compartment if conjugated EM were administered [22]. This study also showed a tendency towards more sustained plasma levels of EM if conjugated estrogens, rather than unconjugated forms, were administered. This study suggests that the conjugated estrogens found in milk are likely to have longer half-lives than non-conjugated estrogens due to first pass metabolism in the liver.

In this study a sensitive and specific LC–MS/MS method was used to measure EM in various commercial milks. These measurements provide a useful tool to assess whether or not the considerable presence of highly reactive catechol estrogens could have a deleterious impact on the typical milk consumer. The estrogens of interest are the highly reactive catechol estrogens (2-OHE_1, 2-OHE_2, and 4-OHE_1), which, to the best of our knowledge, have never been quantified before in any form of milk. Numerous amounts of data have been generated to show that catechol estrogens are strong promoters for carcinogenesis [1]. The consistently high levels of catechol estrogens found in our analysis of whole, 2%, skim, and buttermilk support the theory that milk consumption may influence cancer risk.

In addition to these EM measurements, we have begun looking at other steroid hormones that may be present in appreciable levels in commercial milk products. Preliminary analysis shows that progesterone levels correlate with the EM levels found in the milks. For example, 2% and whole milk display the highest levels of concentration in the free-form of EM (excluding buttermilk). Likewise, 2% and whole milk showed the highest concentration of progesterone in the unconjugated biologically active form. We are currently refining our methods to more accurately and precisely quantify levels of progesterone in these (whole, 2%, skim, and buttermilk) milks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.jchromb.2009.01.032.

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