JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Naturally Occurring Estrogens in Processed Milk and in Raw Milk (from Gestated Cows)

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The occurrence of the steroid hormones estrone (E_1), 17α-estradiol (αE_2), 17β-estradiol (βE_2), and estriol (E_3) in processed bovine milk with different fat contents and in raw milk from (non)gestated cows was investigated. Following liquid extraction, optional enzymatical deconjugation, C_{18} solid-phase extraction, and derivatization, estrogens were analyzed using liquid chromatography−tandem mass spectrometry (LC-MS/MS). Free and deconjugated E_1 (6.2−1266 ng/L) was the major estrogen followed by αE_2 (7.2−322 ng/L) and βE_2 (5.6−51 ng/L), whereas E_3 was detected regularly at the detection limit of 10 ng/L. The lowest and highest concentrations were determined in raw milk from nonpregnant and from cows in the third trimester of gestation, respectively. The estrogen concentration in processed milk coincides with that of raw milk between first and second trimesters, reflecting the contribution of lactating pregnant cows to the final consumable product. The daily intake of total investigated estrogens through milk is 372 ng, which is dramatically more than currently recognized.

KEYWORDS: Endocrine disruptors; food safety; steroid hormones; estrogenic activity in food; veterinary public health

INTRODUCTION

Several studies have investigated the adverse effects of endocrine-disrupting environmental contaminants on human and animal (reproductive) health and the possible role of these substances in human carcinogenesis. Hitherto, environmental compounds with an estrogen(-like) action have attracted the most attention. Remarkably, only a few studies have addressed the exposure of consumers to naturally occurring steroids with endocrine-disrupting potential in food of animal origin (*1*, *2*). Compared to environmental endocrine disruptors, such as mycoestrogens (*3*) and phytoestrogens (*4*, *5*), estrogenic steroids, such as estrone (E_1), 17β-estradiol (βE_2), and estriol (E_3), produced endogenously by the food-producing animal, possess a much more profound estrogenic activity. These substances should, therefore, be considered in the discussion on endocrine disruptors in food as well.

A dramatic increase of estrogen-dependent diseases, such as testicular, breast, prostate, ovarian, and corpus uteri cancers, has been recognized (*6*−*9*). Very recently, a strong epidemiologic correlation between female cancer incidence rate and food consumption, in particular, that of meat and dairy products, was revealed (*8*). For example, the intake of milk and cheese correlated closely with ovarian and corpus uteri cancer. It was suggested that dairy-borne estrogens have played a role in the carcinogenesis in affected women (*8*).

The concentration of naturally occurring estrogens in food largely depends on the type of animal product and the food-producing species and its gender, age, and physiological condition (*10*). Milk is considered to be a rich source of steroids, including estrogens (*11*). Milk-borne steroid hormones originate from their active (transport) and passive (diffusion) passage over the blood−milk barrier (*12*). In addition, mammary biosynthesis of βE_2, but not of E_1 or 17α-estradiol (αE_2), has been shown in cow, goat, and sheep (*13*, *14*). Indeed, the βE_2 concentration was higher in the mammary drainage than in the peripheral circulation, in particular, in high-yielding cows (*14*). It has, however, not been investigated whether mammary-secreted βE_2 contributes to the occurrence of this hormone in milk.

In a Western diet, milk is produced predominantly by lactating cattle, and approximately 75% of this milk originates from pregnant cows (*15*). In mammals, gestation is under the control of relatively high levels of steroid hormones, including estrogens, and correspondingly high levels of milk-borne estrogens can be expected. Indeed, 60−80% of the dietary intake of estrogens originates from milk and other dairy foods in the Western world (*16*).

Toxicological studies in animals and epidemiological studies in the human population have indicated that βE_2 and some of its hydroxyl metabolites could be categorized as carcinogens (*17*). Information, however, on the occurrence of these substances in dairy products is scarce, and scientific evidence for their relationship with malignancies in frequent consumers does

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10.1021/jf061972e CCC: $33.50
Published on Web 11/22/2006

Table 1. Published Estrogen Steroid Concentrations in Bovine Milk (Adapted from Reference *21*)

source	hormone (μg/L) E_1	βE_2	E_3	year of publication
milk from market (3.5% fat)	0.13	<0.02		1998
whole milk from market	0.034	0.006	0.009	1979[a]
raw milk	0.03–0.12	0.01–0.06		1977
raw milk	0.01	0.03		1977
raw milk	0.056	0.01		1979[a]
milk from cyclic cow	0.04	0.04		1975
pregnant cow				
first trimester	0.06	0.09		1975
second trimeste	0.04	0.05		1975
third trimester	0.10	0.05		1975

[a] Reference *22*.

not exist. Hitherto, concentration data have predominantly originated from affinity assay analyses (**Table 1**). Because affinity assays do not provide molecular structure identification of the captured molecule, little or no information is available on the relevant (conjugated) estrogen variants in milk. This study was initiated to determine free and conjugated E_1, αE_2, βE_2, and E_3 in processed milk, in raw tank milk, and in raw milk produced by cows at different gestation trimesters.

MATERIALS AND METHODS

Reagents and Chemicals. Estrone (E_1), 17α-estradiol (αE_2), 17β-estradiol (βE_2), estriol (E_3), and 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride) were purchased from Sigma-Aldrich (St. Louis, MO). Estrogens were each dissolved in methanol to obtain 1.0 mg/mL, and each stock solution was stored at −20 °C. Working solutions were prepared at appropriate concentrations and stored at 4 °C. A standard solution containing 10 ng/mL of each individual estrogen was prepared from this working solution and was stored at 4 °C as well.

Methanol, acetone, acetonitrile, hexane, and dichloromethane (DCM) were of HPLC grade and obtained from J. T. Baker (Deventer, The Netherlands). A β-glucuronidase/sulfatase-containing extract from *Helix pomatia* was obtained from Sigma-Aldrich.

Analytical grade acetic acid was from Merck (Darmstadt, Germany). Solid-phase extraction (SPE) cartridges C_{18} (500 mg; 3 mL) were purchased from J. T. Baker, whereas smaller C_{18} SPE cartridges (100 mg; 1 mL) were obtained from Varian (Bergen op Zoom, The Netherlands). Water was processed through a Milli-Q purification system (Millipore, Bedford, MA).

Raw milk was collected and pooled from four nonpregnant Holstein × Friesian cows housed at the experimental farm facility of the Faculty of Veterinary Medicine (Utrecht University, Utrecht, The Netherlands). Raw tank milk samples and pooled raw milk from Holstein × Friesian cows at their first (five animals), second (five animals), and third trimester (five animals) of gestation, as determined from the moment that they were inseminated, were also obtained from this farm facility. Milk samples were frozen at −20 °C until analysis. Processed milk containing 3.5% (m/v), 1.5% (m/v), or no fat produced by different milk-processing companies was bought in local grocery stores.

Hydrolysis and Sample Preparation. Prior to extraction, frozen milk samples were thawed in a water bath at ambient temperature. Milk samples (10 mL) were transferred into glass tubes (16 mm i.d. × 150 mm). To determine the total concentration, that is, that of the free and deconjugated forms, of estrogens in milk samples, 2 mL of 2% (v/v) acetic acid was added to the homogenized samples and the mixture was treated with 500 units of β-glucuronidase/sulfatase dissolved in 100 μL of 40 mM acetate buffer at pH 5.3. Following vortex mixing, incubation was performed overnight at 37 °C (*18*).

Quality control samples were prepared by spiking milk samples with a standard mixture of estrogens to final concentrations of 0.12, 0.25, 0.50, and 1.0 ng/mL for each estrogen variant. Following vortex mixing for 15 s, samples were homogenized for 30 min in an ultrasonic water bath.

Cleanup Procedures. A volume of 10 mL of a mixture of methanol and water (8:2, v/v) was added to nonspiked, spiked, or β-glucuronidase/sulfatase-treated milk samples. Each suspension was vigorously mixed for 10 min at 500 rpm on a platform shaker and centrifuged at 2000*g* for 10 min at 4 °C. The upper fat layer was discarded, whereas the underlying supernatants were transferred to fresh tubes. The analyte-containing fraction was mixed with 10 mL of hexane and centrifuged at 3500*g* for 10 min. Following the careful removal of the hexane layer, 15 mL of DCM was added to the residue. After 15 s of vortex mixing and 10 min of shaking at 500 rpm on a platform shaker, samples were centrifuged at 2000*g* for 10 min at 4 °C. The lower layer was transferred into a fresh glass tube, and extraction of the supernatant with 15 mL of DCM was repeated. The DCM phases were combined, homogenized, and dried under a stream of N_2 gas. The residue was reconstituted in 0.5 mL of methanol, and following vortex mixing, 9.5 mL of water was added. After homogenization, the solution was passed carefully through a C_{18} SPE column (500 mg; 3 mL), which was activated and conditioned with 5 mL of methanol and 5 mL of water, respectively. The analyte-containing column was washed with 5 mL of water and dried using N_2(g). The hormones were eluted with 4 mL of methanol, and the solvent of the collected fraction was evaporated under a stream of N_2(g) at ambient temperature.

Derivatization of Estrogens. Calibration standards were prepared from working solutions and dried under a stream of N_2(g) so that tubes contained a mixture of 0, 0.25, 0.5, 1.0, 2.0, and 5.0 ng of each steroid variant. Dried calibration standards and milk extracts were reconstituted in 0.25 mL of 100 mM $NaHCO_3$ at pH 10.5. Estrogens were derivatized by the addition of 0.25 mL of 1 mg/mL dansyl chloride in acetone followed by incubation at 60 °C for 3 min.

After derivatization, samples were purified over C_{18} SPE columns (100 mg; 1 mL), which were first activated with 1 mL of methanol and then conditioned with 1 mL of water. Columns were washed with 1 mL of water followed by 1 mL of a mixture of acetonitrile and water (40:60, v/v), and finally analytes were eluted with 1 mL of methanol. Collected eluates were dried immediately under N_2(g), and particulate material was resolved in 0.2 mL of a mixture of acetonitrile and water at a ratio of 40:60 (v/v). Samples were transferred to autosampler glass vials equipped with 200 μL inserts and sealed.

LC-MS/MS. For LC-MS/MS analysis, 10 μL aliquots were injected into an HPLC system consisting of two HPLC pumps (PE200 series), an autosampler (PE200 series), and an API-3000 MS detector equipped with an electrospray interface. All of these instruments were purchased from Applied Biosystems (Foster City, CA). The HPLC column was a Luna C_{18} (150 × 2.0 mm, Phenomenex, Torrance, CA), which was eluted with mobile phase A consisting of a mixture of acetonitrile, water, and formic acid at a ratio of 40:60:0.4 (v/v/v) and with mobile phase B consisting of acetonitrile, water, and formic acid at a ratio of 90:10:0.4 (v/v/v). The flow rate was set at 200 μL/min using a linear gradient run as follows: 100% A for 1 min, to 100% B in 9 min, and finally 100% B for 14 min. The HPLC column was equilibrated with 100% A for 10 min prior to the next injection.

The electrospray interface of the MS was operated at a voltage of 5500 V and a source temperature of 400 °C. The entrance, declustering and focusing potentials were set at 10, 76, and 260 V, respectively. Nitrogen was used as curtain gas (setting 10). Tandem MS analysis was performed in positive multiple reaction monitoring (MRM) mode. The collision energy was set at 50 V. The transitions were reported for E_1, αE_2, βE_2, and E_3 (**Table 2**). The LC-MS/MS instrument was controlled by the Analyst software package (version 1.4.1, Applied Biosystems).

Evaluation of Data. Quantification of concentrations was carried out using external standards. Concentrations were then corrected for the recovery of the respective analyte from the milk matrix. The limit of detection (LOD) for each analyte was established by determining the signal-to-noise ratio at 3.

In the case of quality controls, evaluation of data was performed following the correction of peak areas for the 'background' signals

Table 2. LC-MS/MS Analysis of Estrogens in Raw Tank Milk[a]

compd	MRM trace (*m*/*z*)	recovery (%)	LOD[b] (ng/L)
E_1	504 → 171	63 ± 12	5
αE_2	506 → 171	65 ± 9	5
βE_2	506 → 171	77 ± 12	5
E_3	522 → 171	51 ± 13	10

[a] Milk was spiked with the listed estrogens. Recoveries from three experiments are given as averaged values ± standard deviations. [b] Limit of detection determined in milk.

stemming from the naturally occurring estrogens. These 'background' signals were obtained by the analysis of nonspiked ('blank') samples.

RESULTS AND DISCUSSION

The presence of estrogens E_1, αE_2, βE_2, and E_3 in raw and processed milk samples was investigated. The application of LC-MS/MS to the analysis of the targeted molecules in a food matrix, such as milk, was hitherto unconventional, and this is to our knowledge the first study using this analytical platform for this purpose.

Preliminary LC-MS/MS analyses of milk samples gave rise to unsatisfactory sensitivity, despite the use of MRM for the most responsive detection of estrogens with the mass spectrometer in the negative mode. In the positive mode, sensitivity was dramatically improved following derivatization of analytes with dansyl chloride (**Figure 1**), as described for ethinylestradiol in plasma samples from rhesus monkey (*19*).

To establish analytical characteristics for each analyte, milk was spiked with a mixture of estrogen variants. In this way, recoveries and LODs for each analyte were determined (**Table 2**). The most intensive parent−product ion transition was used for quantification, which was the fragmentation of the molecular ion of E_1, αE_2, βE_2, and E_3 to product ion at *m*/*z* 171. This value reflects a 5-dimethylaminonaphthalene residue originating from the dansyl moiety. In this way, dansylated estrogens gave LODs of 5 ng/L for E_1, αE_2, and βE_2 and 10 ng/L for E_3 in milk for the overall method from sample cleanup to LC-MS/MS analysis (**Table 2**). It must be noted that the monitoring of the transition to the dansyl derivative at *m*/*z* 171 may not increase the specificity of the analysis, but was necessary to acquire satisfactory sensitivity. Transitions of the molecular ion to structural fragments of the carbon skeleton of monitored estrogens were predominantly failing to obtain an acceptable sensitivity.

The recoveries for E_1, αE_2, βE_2, and E_3 were 63, 65, 77, and 51%, respectively (**Table 2**). Although these recoveries may be considered to be low, they were regarded as acceptable in the presented multiresidue method analyzing four estrogen variants simultaneously in a single LC-MS/MS run. In fact, the values are within the range of −50 to 20% for the minimum trueness of quantitative methods according to EU Commission Decision 2002/657 (*20*). Regression analysis of standard curves of each dansylated estrogen showed reproducible squared correlation coefficients of >0.991. Following analysis, milk samples were spiked occasionally with dansylated standards and analyzed again only for the purpose of confirmation of the identity of the detected estrogen.

Dansylated E_1, αE_2, and βE_2 were successfully detected in milk extracts (**Tables 3**−**5** and **Figure 2**). The sensitivity of the method was, however, in most cases unsatisfactory for the detection of E_3. Nonconjugated αE_2 was detected in raw milk but occurred apparently at a concentration below the LOD in processed milk, Such "free" forms of estrogens were determined by omitting incubation of milk samples with glucuronidase and sulfatase (**Table 3**). Free E_1 and βE_2 were found in raw milk as well as in processed milk (**Tables 3** and **5**). The measured E_1 and βE_2 concentrations in these products were in accordance with other studies (cf. **Table 1**).

As expected on the basis of the fat content in raw milk (3.4−5.1%), processed milk with the highest fat content (3.5%) contained concentrations of lipophilic E_1 and βE_2 (**Table 3**) comparable to those in raw milk (**Table 5**). Processing of the milk seemed to have little effect on the final concentration of the estrogens in consumption-ready milk (*11*, *21*). Going from 3.5% fat to 0% fat (skim milk) content of the processed milk, the concentrations of E_1 and βE_2 decreased in accordance with other results (*11*, *22*). It should be noted here that 0% fat was indicated on the label of the bought milk product but in fact the milk has a residual fat content.

The analysis of full-fat, processed milk was performed twice (**Tables 3** and **5**). The difference in these samples was the season in which they were obtained, namely, in late autumn (**Table 3**) and in late spring (**Table 5**). This seasonal difference may give an explanation for the differences in concentrations found in these samples. Free E_1 concentrations were comparable, but the spring βE_2 concentration was a fourth of that found in the autumn samples. Similarly, the free βE_2 content in raw tank milk collected in late autumn was significantly higher than that of the sum of free and deconjugated βE_2 in raw tank milk collected a few months later (**Table 5**). This suggested that the seasonal influence on the final βE_2 content in milk needs further investigation, as such an effect has not been described before. Although a single observation, another interesting result is that organically produced milk contained considerably more αE_2 (duplicate measurements: 101 and 104 ng/L) compared to all other sampled conventionally produced milks in this study.

Pooled raw milk collected from nonpregnant cows ($n = 4$) and from pregnant cows at their different trimesters of gestation ($n = 5$ for each trimester) was analyzed as well (**Table 3**). Free estrogens are markedly elevated in the second half of the pregnancy. In particular, E_1 increased, as expected (*23*), by a factor 19 in concentration compared to values from nonpregnant cows. The free forms of αE_2 and βE_2 rose less spectacularly by factors of 6.5 and 3.7, respectively. Interestingly, a high portion of milk from pregnant cows in market-available 3.5% fat milk (*15*) seems to be reflected in the concentration of free estrogens. The consumable-ready milk is comparable with raw milk collected between the first and second trimesters of pregnancy, but not with milk from nonpregnant cows (cf. **Table 3**).

The cumulative concentration of free and enzymatically deconjugated estrogens in the third trimester (1639 ng/L) was >27 times higher than that in milk of cows in their first trimester of pregnancy (60 ng/L) (**Table 4**). Compared to raw tank milk (**Table 5**) this factor was 16. This observation is of relevance as current farm practices driven by economic factors tend to shorten nonpregnancy intervals. In other words, consumable milk may consist increasingly of milk from pregnant cows, introducing higher estrogen concentrations in the final food product. Of the determined estrogens, 87−92% in full-fat milk (**Table 5**) and up to 91% (**Table 4**) in pregnant cow's milk occurred as conjugated variants of E_1, αE_2, and βE_2. This fraction is comparable to human milk, in which >90% of the estrogens (E_1, βE_2, and E_3) were found to be conjugated (*24*).

The daily intake of βE_2 through milk is believed to range from 45 to 135 ng on the basis of 1.5 L of milk (*2*). This study shows that these data are not complete as analysis of the free

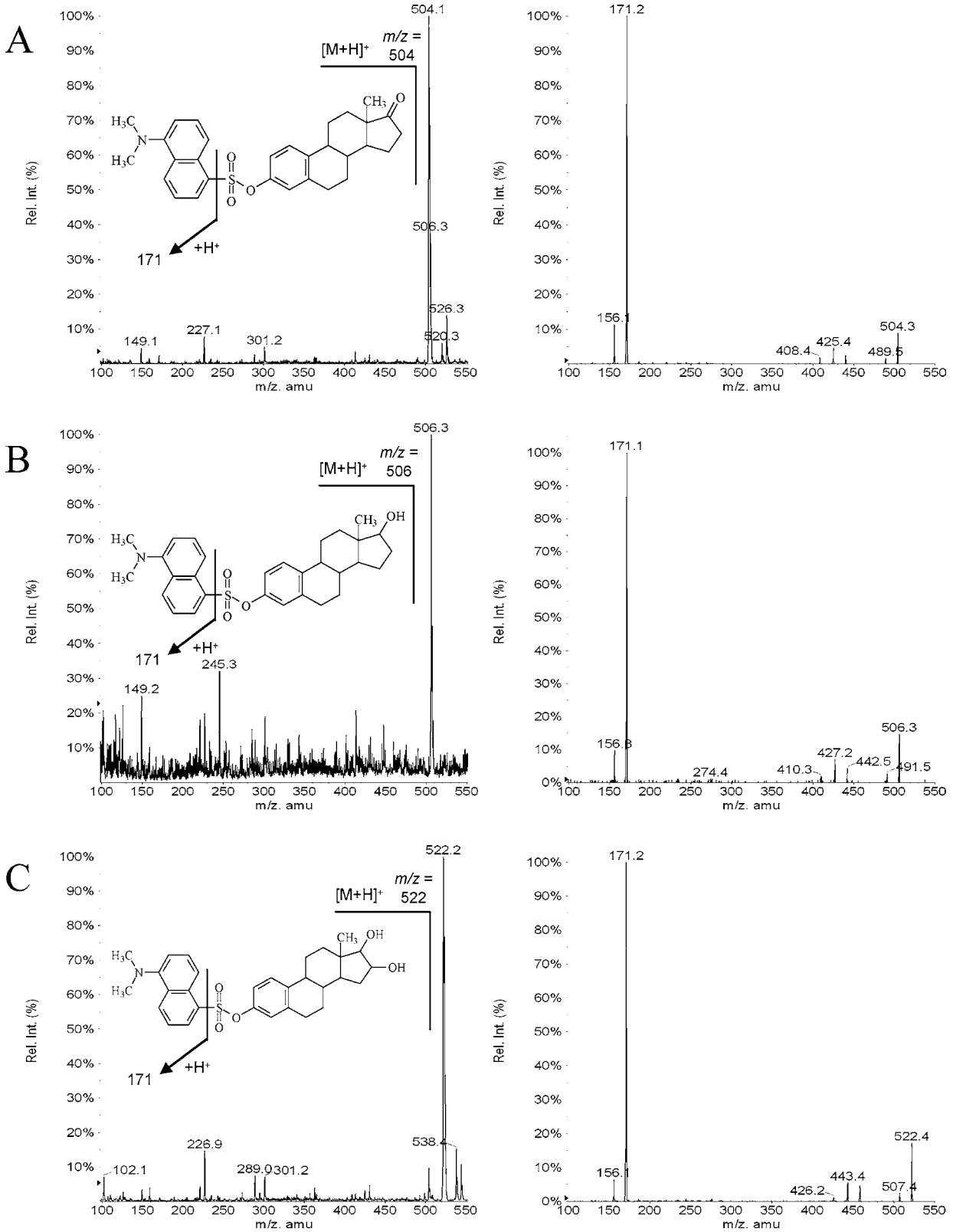

Figure 1. Q1 (left-column panels) and product-ion (right-column panels) mass spectra of dansylated reference estrone (**A**), estradiol (**B**), and estriol (**C**), respectively.

and released estrogens gave an averaged final concentration of 248 ± 41 ng/L in the conventionally produced processed full-fat milks (**Table 5**). This would thus contribute to a daily exposure of 372 ng of estrogens on average through milk on the basis of the same consumption volume.

When the individual compounds were examined, βE_2 rose by a factor of 2.8 going from the first to the third trimester of pregnancy, whereas the αE_2 content increased approximately 10-fold and that of E_1 as much as 160 times (**Figure 2**). It must be noted here that the E_1 concentration in milk of the first trimester was found unexpectedly lower in enzyme-treated (7.9 ng/L) than in non-enzyme-treated (9.2 ng/L) milk.

Similar to human milk (60%), the relative contribution of conjugated E_1 was as high as 67% in raw milk and 73% in

Table 3. Levels of Nonconjugated (Free) E_1, αE_2, βE_2, and E_3 in Processed Milk Samples Containing Various Concentrations of Fat and in Bovine Milk from Nonpregnant Cows and from Lactating Cows, Collected at Different Trimesters of Gestation[a]

	concentration (ng/L)						
	processed				raw: trimester of gestation		
compd	0% fat[b]	1.5% fat	3.5% fat	raw: nonpregnant	first	second	third
E_1	8.2 ± 0.7[c]	17.1 ± 0.9	20 ± 4	6.2 ± 1.2	9.2 ± 0.3	57 ± 11	118 ± 17
αE_2	<LOD[d]	<LOD	<LOD	7.2 ± 1.7	12 ± 4	17.1 ± 1.1	47 ± 2
βE_2	10.3 ± 1.4	13.9 ± 1.8	20.6 ± 1.5	5.6 ± 1.2	10 ± 2	20.4 ± 1.4	21 ± 3
E_3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

[a] Three bottles of each type of processed milk were bought and per type combined for sampling. Each sample was analyzed in triplicate. [b] Concentration of fat in processed, market-available milk as indicated on the label of the product. [c] Averaged concentration ± standard deviation. [d] <LOD, not detected.

Table 4. Concentrations of the Sum of Free and Deconjugated E_1, αE_2, βE_2, and E_3 in Bovine Milk Collected at Different Trimesters of Pregnancy from Lactating Cows[a]

	trimester of gestation		
compd	first	second	third
E_1	7.9 ± 0.7[b] (–)[c]	452 ± 66 (87%)	1266 ± 38 (91%)
αE_2	33 ± 7 (64%)	84 ± 4 (80%)	322 ± 35 (85%)
βE_2	18.6 ± 0.2 (46%)	51.4 ± 2.7 (61%)	51.2 ± 1.5 (59%)
E_3	<LOD[d]	<LOD	<LOD

[a] Each sample was analyzed in triplicate. Concentrations are given in ng/L. The degree of conjugation is indicated in parentheses. [b] Averaged concentration ± standard deviation. [c] Ratio could not be given as the concentration of the sum is smaller than that of the free (nonconjugated) compound. [d] <LOD, not detected.

Table 5. Concentrations of the Sum of Free and Deconjugated E_1, αE_2, βE_2, and E_3 in Raw Tank Milk and in Processed Consumable-Ready Full-Fat (3.5%) Bovine Milk from Different Producers (Coded by Numbers)[a]

		concentration (ng/L)				
product/ producer	enzymatic treatment	E_1	αE_2	βE_2	E_3	conjugation (%)
tank milk[b]	–	23.4 ± 1.9[c]	na[d]	20 ± 2	nd[e]	58
	+	93 ± 4	nd	11.5 ± 0.6	nd	
1	–	23	nd	5	nd	87
	+	162	44	10	nd	
2	–	22	nd	nd	nd	92
	+	208	36	10	10	
3	–	27	nd	5	nd	85
	+	174	30	9	nd	
4	–	24	nd	nd	nd	92
	+	243	44	11	obs[f]	
5 (organic)	–	29	nd	nd	nd	92
	+	222	102	12	12	

[a] A single sample is indicated as milk from an organic production system (no. 5); the other samples were taken from conventionally produced milk. Given concentrations for processed milk are the average of results from measurements in duplicate. Raw tank milk samples were analyzed in triplicate. [b] Raw tank milk. [c] Averaged concentration ± standard deviation. [d] Not analyzed. [e] Not detected. [f] Present, but not quantifiable, as the signal was obscured by an interference.

processed milk of all detected (conjugated) estrogen variants in raw tank milk (**Table 5**). The development of estrone sulfate conjugate in bovine milk has been demonstrated as an indicator of viable conceptus (*25*, *26*), and also in caprine milk the concentration of estrone sulfate rose during pregnancy (*27*). The concentration of conjugated E_1, including sulfated E_1, at 1.3 μg/L in the third trimester (**Table 4**) coincides with the maximum of 1 μg/L sulfated E_1 conjugate for milk from cows at days 220−240 of gestation (*24*).

Clearly in contrast to our findings and that of others, the αE_2 variant was also reported to be the main estrogen in bovine milk followed by estrone and βE_2 (*28*). Although the concentration of βE_2 found here is in accordance with that reported (*11*), our results for E_1 differ from other published data (summarized in **Table 1**). Data are, however, often difficult to assess because levels of hormones were expressed in different units, or different conjugated forms of the hormones were measured, or otherwise different assays for different samples from different breeds with different physiological status, feeding regimen, etc., were used (*2*). In fact, most published results relied on analyses performed in the 1970s using radio-immunoassays (RIA) often without deconjugation of the estrogens (see, for example, refs *22*, *25*, and *29*). Most studies also did not include the analysis of E_1 and αE_2.

The selection of lactating races and improvement of the zootechnical circumstances in the farming of animals is ongoing, and since the 1970s the production volume of milk per cow has much increased. This increase is likely the result of changes in the endocrinological system of the lactating cow and may thus have in summa influenced the secretion and molecular fingerprint of hormones in milk. In light of the suggested link between dairy consumption and carcinogenesis in humans, it is, therefore, meaningful to continue the monitoring of free and conjugated hormones in modern milk, which is a universal and important food product.

It should be realized that conjugated estrogens are not biologically active, but that conjugates can be cleaved in the human gut to free the estrogens to their corresponding active form. This release is accomplished by bacterial sulfatases and by bacterial and/or endogenous glucuronidases (*29*). The estrogenic activity of the detected estrogens is in the following order: $\beta E_2 > E_1 \gg E_3 > \alpha E_2$ (*21*). Furthermore, whereas αE_2 does not seem to be carcinogenic and E_3 may have protective properties (*22*), E_1 has been shown to be a strong carcinogen in hamster kidney (*30*). As E_1 and βE_2 represented at least 80% of the total amount of measured estrogens, except in milk from nonpregnant cows (44%) and in organically produced milk (67%), a significant amount of estrogenic and carcinogenic activity may be released from consumed milk in the human gut.

It should be noted, however, that whether such biological effects from the consumption of bovine milk will occur in the human population cannot be concluded on the basis of the results presented in this study. To draw such conclusions would require an assessment of the total exposure toward estrogens, bioavailability, kinetics, and dynamics of metabolism (especially of the first liver passage), sensitivity of tissues toward estrogens, etc. Nevertheless, over the past recent years, concern has been raised about the possible adverse effect of milk-borne estrogens by epidemiologically found correlations, but without scientific evidence for a relationship. The presented results may, therefore, contribute to an advanced assessment of the health risks of

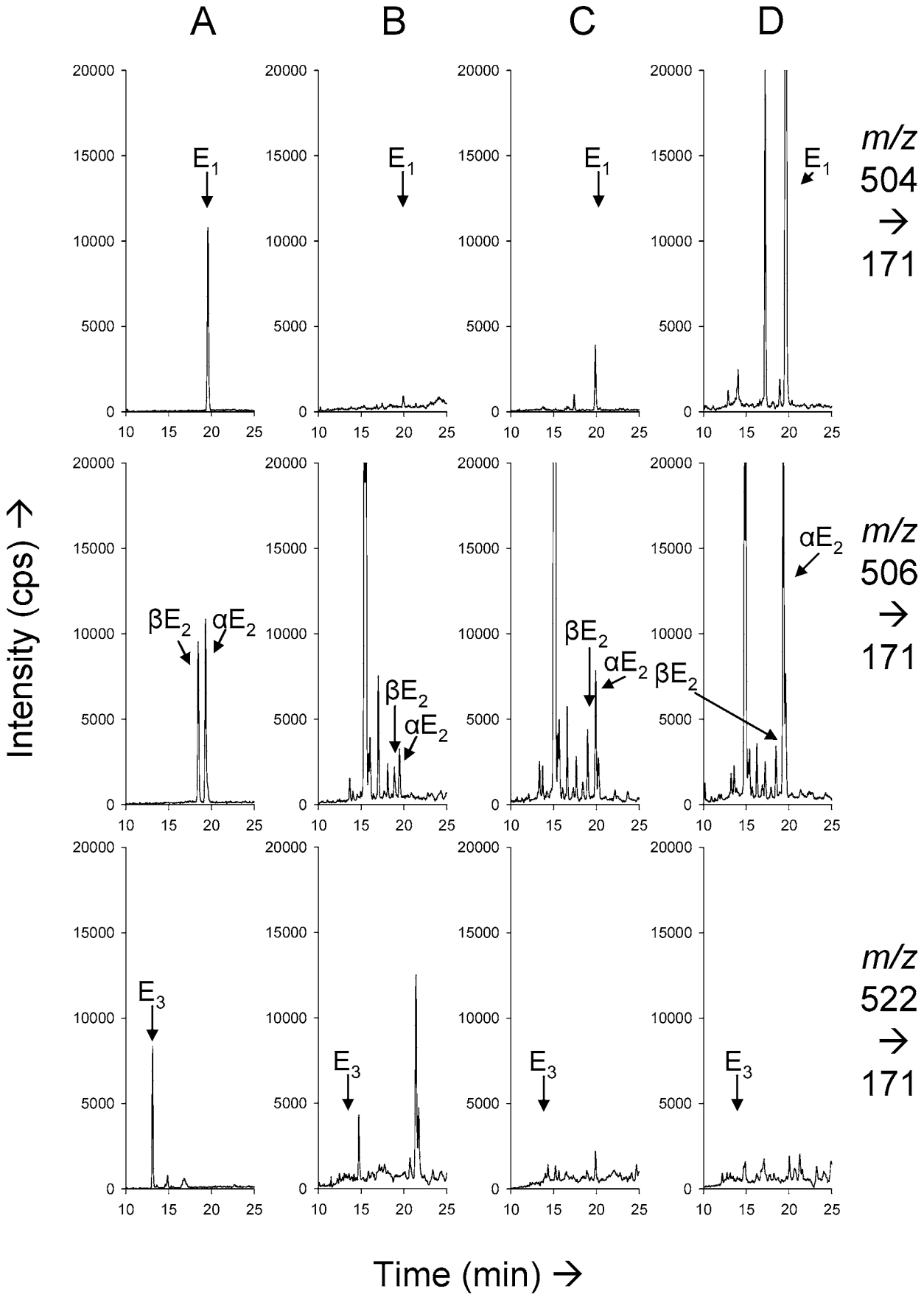

Figure 2. LC-MS/MS chromatograms of E_1, αE_2, βE_2, and E_3 in reference solution (**A**) and in raw milk from cows in their first trimester (**B**), second trimester (**C**), and third trimester (**D**) of gestation. The following transitions were monitored: *m*/*z* 504 → *m*/*z* 171 (E_1, upper panel), *m*/*z* 506 → *m*/*z* 171 (αE_2 and βE_2, middle panel), and *m*/*z* 522 → *m*/*z* 171 (E_3, lower panel).

estrogens occurring in dairy products derived from cows, in particular, from those in their late gestation.

ABBREVIATIONS USED

αE_2, 17α-estradiol; βE_2, 17β-estradiol; E_1, estrone; E_3, estriol; DCM, dichloromethane; EU, European Union; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography−tandem mass spectrometry; LOD, limit of detection; MRM, multiple reaction monitoring; RIA, radioimmunoassays; SPE, solid-phase extraction.

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Received for review July 14, 2006. Revised manuscript received September 25, 2006. Accepted October 6, 2006.

JF061972E