J. Dairy Sci. 87:2331–2336

Technical Note: Measurement of Total Estrone Content in Foods. Application to Dairy Products

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ABSTRACT

Estrone is a powerful growth-inducing hormone that is present in milk, mainly in the form of fatty acid esters, at concentrations that promote growth in experimental animals. We present here a method useful for the measurement of this natural hormone in foods and applied it to several common dairy products. Samples were frozen, finely powdered, and lyophilized then extracted with trichloromethane/methanol; the dry extract was saponified with potassium hydroxide. The free estrone evolved was extracted with ethyl acetate and was used for the estimation of total estrone content through radioimmunoassay. Application of the method to dairy products showed high relative levels of total estrone (essentially acyl-estrone) in milk, in the range of 1 μM, which were halved in skimmed milk. Free estrone levels were much lower, in the nanomolar range. A large proportion of estrone esters was present in all other dairy products, fairly correlated with their fat content. The amount of estrone carried by milk is well within the range, where its intake may exert a physiological response in the sucklings for which it is provided. These growth-inducing and energy expenditure-lowering effects may affect humans ingesting significant amounts of dairy products.
(**Key words:** dairy product, milk, estrone, acyl-estrone)

Estrone is a powerful growth factor (Lacroix et al., 2002) that is closely related to estradiol, the main estrogenic hormone, but estrone is essentially devoid of direct estrogenic effects because of its low affinity for the estrogen receptors (Cabot et al., 2001). The administration of low doses of free estrone or its fatty esters to experimental animals results in increased growth and metabolic efficiency (Sanchis et al., 1996; Remesar et al., 1999). Estrone is synthesized in large amounts during pregnancy, increasing its urine excretion by several orders of magnitude (Berg and Kuss, 1992). It may be assumed that the induction of growth, fat accumulation, and decreased energy expenditure that characterize pregnancy and its concurrent fetal growth may be, at least partially, due to the estrone increases in the maternal-fetal unit (de Hertog et al., 1975). Nevertheless, despite a high and continued fetal exposure to estrone, the hormonal and developmental normalcy of male neonates is proof of the lack of significant estrogenic effects of estrone on the fetus, confirming that estrone is a nonestrogenic growth factor.

The energy demands of pregnancy (Prentice et al., 1989) are, however, small when compared with the energy demands of lactation (Remesar et al., 1981), as the energy provided by the milk not only must allow growth but also fulfill the high energy demands of thermogenesis linked to the initial stages of independent life (Girard and Ferré, 1982). The high-energy needs of the sucklings must be entirely supplied by the dam; this is accomplished in part thanks to a higher metabolic efficiency, but most of the energy needed is provided by increased energy intake and the use of its own reserves (Remesar et al., 1981). The high cost of this energy for the dam requires that the sucklings develop a high degree of efficiency in the utilization of the nutrients, preventing wasted energy. The continued exposure of the newborn to maternal estrone through milk may help to fulfill this purpose.

The free estrone content of milk is very low (Hartman et al., 1998) and in the range of that found in plasma (Sanchis et al., 1997a). However, milk contains very high amounts of estrone esterified with fatty acids, essentially oleoyl-estrone (Remesar et al., 1999).

Oleoyl-estrone is a lipostatic signal synthesized by adipose tissue (Sanchis et al., 1997b). Its pharmacological administration to rats results in the loss of body fat, sparing protein (Sanchis et al., 1996; Grasa et al., 2001) and thus reverting the effects of free estrone (Sanchis et al., 1996). However, the levels of oleoyl-estrone in milk are under the threshold of its observed slimming effects, which take place at pharmacological doses (Remesar et al., 1999). Young rats fed the hor-

Received March 1, 2004.
Accepted March 16, 2004.
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mone ester at concentrations close to those found in milk have been found to increase their body mass significantly compared with controls (Tang et al., 2001).

Continued milk consumption by adult populations has been associated both with increased and decreased (Barr, 2003; Phillips et al., 2003) obesity rates, although its enhancement of growth in the young has been well established (Pereira et al., 2002). It remains to be ascertained, however, in which proportion these effects directly relate to the presence of estrone in milk and which part is only a consequence of the high-energy and high-quality mix of nutrients that the milk provides. We have intended here to establish a reliable method for the analysis of total estrone in dairy products and to determine which is their potential dietary contribution to our daily consumption of this growth-enhancing hormone.

All dairy products used were commercial brands widely available in Spanish retail outlets. Five different samples of each product were used in the analyses (3 in the case of cheeses).

Estrone (Sigma, St. Louis, MO) and oleoyl-estrone (Oleoyl-estrone-Developments SL, Barcelona, Spain) were used as standards and to spike the products analyzed as internal standards. Tritium-labeled oleoyl-estrone was synthesized from labeled estrone (Perkin-Elmer Life Science, Boston, MA) (Ardévol et al., 1997) and purified by thin layer chromatography, achieving a specific activity of 2.4 Tbq/nmol. All reagents and solvents used were of analytical purity. Polyclonal antibodies against estrone were obtained from ICN Biomedicals (Costa Mesa, CA).

The method described followed the general outline of that used for the measurement of acyl-estrone in plasma (Ardévol et al., 1997), which has also been applied earlier to the analysis of a number of foods (Remesar et al., 1999). It consists in the initial extraction of estrone and acyl-estrone from the samples using trichloromethane:methanol (Folch et al., 1957), followed by saponification with hydroalcoholic KOH, and organic phase extraction of the estrone evolved, which was later measured using a standard radioimmunoassay method.

Samples of about 1.5 g of solid products were weighed, frozen in liquid nitrogen, and powdered to a fine powder with mortar and pestle under liquid nitrogen. After all nitrogen had evaporated, the frozen samples were completely dehydrated for 24 h in a lyophilizer (model Cryodos; Telstar, Terrassa, Spain). The dry powder was weighed again and immediately introduced in Teflon-lined, screw-cap tubes containing 10 mL of a trichloromethane:methanol (2:1 vol/vol) mixture. The samples were extracted for 24 h in an orbital shaker at room temperature. They were filtered through a glass-wool pad, and 2 mL of 9 g/L NaCl were added. The tubes were left again for 30 min in the orbital shaker. The phases were then allowed to separate, and the aqueous phase was discarded. The initial volume was reconstituted with pure methanol.

Liquid samples (i.e., milk, about 1 g) were frozen using liquid nitrogen and lyophilized. The resulting dry powder was weighed and introduced in Teflon-lined, screw-cap tubes following the same procedure outlined for solid samples.

An aliquot of 1 mL of the lipid extract was brought to dryness under a gentle stream of nitrogen at 55°C. The residue was re-dissolved in 3 mL of trichloromethane:methanol (2:1), and the tubes were left for 2 h in an orbital shaker. Then, 1 mL of 4 m*M* $MgCl_2$ was added, and the tubes were shaken for 15 min. The phases were separated by centrifugation at 1000 × *g* for 15 min at 10°C, discarding the aqueous phase. The organic phase was quantitatively transferred to another tube, and the solvent was evaporated at 55°C under a gentle stream of nitrogen. The residue was dissolved in 1 mL of 0.4 *M* KOH in ethanol:water (25:2 vol/vol). The tubes were vortexed and maintained at 85°C for 20 min with occasional vortexing. Then, the tubes were allowed to cool, and 1 mL of water and 2 mL of ethyl-acetate were added to each tube; they were vortexed for 1 min and centrifuged for 10 min at 1000 × *g*; the extraction with 2 mL ethyl-acetate was repeated once. The ethyl-acetate fractions were combined in another tube; the solvent was then evaporated at 55°C under a nitrogen stream. The residue was dissolved in 2 mL of ethanol with—eventually—the use of a glass rod. Aliquots of 0.020 to 0.100 mL were taken from the purified extract and brought to 1 mL with 0.01 m*M* phosphate buffer (pH 7.4) containing 0.01 g/L gelatin (Sigma); small amounts of ethanol were added when necessary to bring its final concentration to a uniform 12.5% in all samples. Aliquots of lipid extract were used to determine the lipid content by gravimetry, after the evaporation of solvent in a heater.

The radioimmunoanalysis was carried out mixing 0.100 mL of the diluted sample, to which 0.100 mL (24 kBq, i.e., 100 fmol per tube) of tritium-labeled estrone (Perkin-Elmer) in phosphate buffer was added; after mixing, 0.100 mL of the antibody preparation in phosphate buffer containing 0.01 g/L gelatin was added. After gentle shaking, the tubes were left for either 24 h at 4°C or 1 h at 37°C. (No differences were observed using either approach.) Then, 0.200 mL of a stirred suspension of 5 g/L activated charcoal (Sigma) and 1 g/L dextran (Sigma) in phosphate buffer were added. The tubes were mixed and left to stand for 20 min at 4°C; then, they were centrifuged at 1500 × *g* for 15

Table 1. Estrone content in several dairy products.[1]

Product	Water (%)	Fat (%)	Free estrone (nmol/kg)	Acyl-estrone (μmol/kg)
Butter				
Butter [*Arias*]	14	83	4.6 ± 1.3	9.27 ± 0.80
Cheese				
Mahó	36	34	5.7 ± 1.3	3.39 ± 0.85
Roncal	21	40	5.0 ± 1.1	2.44 ± 0.12
Manchego	39	29	2.8 ± 0.3	2.33 ± 0.18
Emmental	26	33	4.2 ± 1.1	2.13 ± 0.10
Cheddar	27	34	1.6 ± 0.3	2.08 ± 0.52
Low fat	33	5	2.5 ± 0.5	1.87 ± 0.14
Parmesan	33	25	2.8 ± 0.6	1.86 ± 0.11
Camembert	41	24	1.8 ± 0.6	1.67 ± 0.09
Burgos	64	11	0.6 ± 0.2	1.60 ± 0.16
Spreading [*Danone Petit Suisse*]	73	14	3.5 ± 0.2	0.25 ± 0.01
Milk				
Whole pasteurized [*Ato*]	88	4	5.4 ± 1.0	1.00 ± 0.12
Half-skimmed [*Ato*]	91	1.6	5.4 ± 1.8	0.66 ± 0.04
Skimmed [*Ato*]	92	0.3	6.4 ± 0.7	0.53 ± 0.04
Yogurt				
Liquid [*Danone Danup*]	85	1.3	5.6 ± 0.9	0.47 ± 0.14
Plain [*Danone*]	91	3	3.2 ± 0.4	0.27 ± 0.11
Other				
Flan [*Danone*]	69	4	6.5 ± 0.6	1.52 ± 0.28
Custard [*Natillas Danone*]	73	4	5.9 ± 1.0	0.35 ± 0.04

[1]Data are the means ± SEM of 6 duplicate samples. The names of commercial brands analyzed are given in brackets.

min at 4°C. The supernatants (aliquots of 0.200 mL) were pipetted into scintillation vials and counted.

In the radioimmunoassay procedure, the samples were analyzed along with a series of standards containing from 0 to 4 pmol of estrone (and the same proportion of ethanol as the samples). A series of blanks was used for the establishment of the inespecific binding values. The data from the standards were fitted to a standard radioimmunoassay curve, which was then used to establish the estrone content of the samples. The calculations were, finally, related to the initial weight of the sample.

The measurement of free estrone was carried out by directly using the trichloromethane:methanol extract in the radioimmunoassay, i.e., by drying aliquots of this phase instead of aliquots of the ethyl-acetate extracts.

The efficiency of the method was tested by including internal standards of labeled oleoyl-estrone (8.51 kBq/μL), dissolved in a small amount of ethanol, added to the samples, and thoroughly mixed just before freezing. This procedure allowed for a global estimation of the recovery of the full procedure.

Using labeled oleoyl-estrone as tracer, we found that the lyophylization step was necessary, as the presence of even small amounts of water in the sample during the extraction procedure resulted in significant losses of label (and, consequently, of product) in the first extractive phase. After complete dehydration, the recovery of added oleoyl-estrone label in the trichloromethane:methanol extracts of the samples was in the range of 87.9% for whole milk and yogurt, and 87.3% for hard cheese. After saponification, the combined recovery was lower: 84.1% for whole milk, 83.1% for yogurt, and 70.3% for hard cheese. The final recovery, after radioimmunoassay was (mean values for 5 samples each) 78.3% for whole milk, 72.7% for yogurt, and 61.2% for hard cheese.

The use of trichloromethane:methanol as extraction phase was found to yield better results than a 20-cycle Soxhlet extraction using pure methanol or ethanol. Several solvents were also tested for the extraction of the estrone released from the alkaline saponification system: hexane, trichloromethane, trichloromethane:methanol, ethyl-ether, and ethyl-acetate. The latter was the one carrying-over lower amounts of soaps and alkali while recovering a large portion of free estrone. Neutralization of the organic phase was unnecessary because the gain in organic phase solubility of the protonized estrone was countered by the higher solubility of free fatty acids evolved from the soaps. The need to minimize the carrying-over of lipids from the original sample was critical, as lipids, i.e., alkaline soaps, resulted in serious interference to the radioimmunoanalysis.

The use of internal standards of unlabelled oleoyl-estrone randomly distributed among samples allowed for a further checking of the efficiency of the analytical

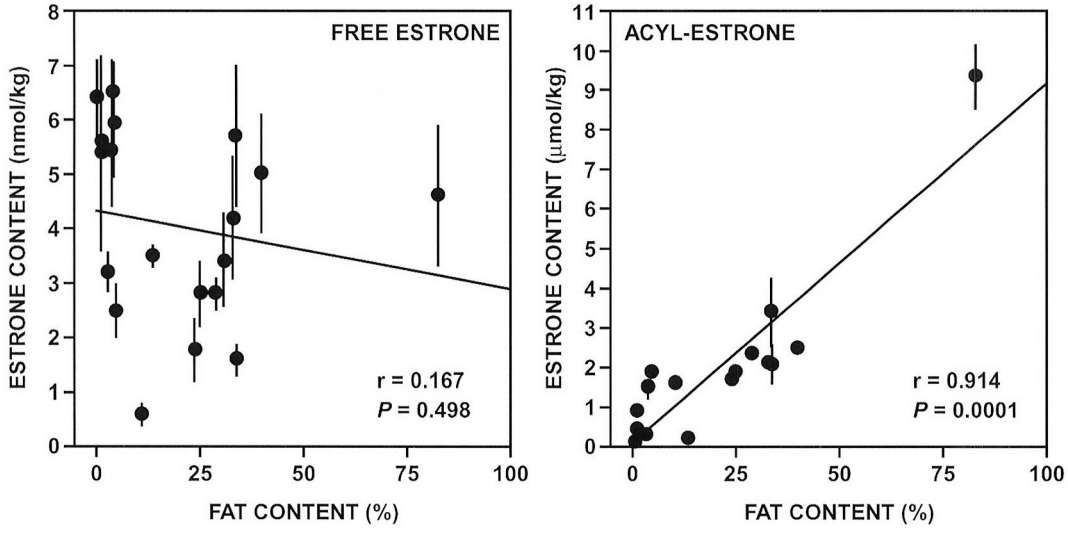

Figure 1. Linear correlations between the percentage of fat in dairy products and a) free estrone content or b) total acyl-estrone content.

system. In all cases, the results obtained were similar to those obtained using the standard procedure outlined here. As a rule, in a given series of analyses, a number of duplicate samples were spiked with internal standards of oleoyl-estrone before dehydration to check for errors and eventually correct any possible artifact caused by the extraction or radioimmunoassay procedures.

Oleoyl-estrone did not interfere with the measurement of free estrone, even when added in large amounts, mainly because its extreme insolubility kept it out of the aqueous solution in which the antigen-antibody reaction took place.

Table 1 shows the mean estrone and acyl-estrone content of commercial samples of dairy products. The dry weights were experimentally measured. The percentage of solids was determined after dessication of the samples in an oven at 110°C for 12 h. Lipid content was analyzed (Folch et al., 1957) by solvent extraction and gravimetry.

Whole milk and most cheeses contained a very large concentration of total estrone. The distribution of free estrone was rather uniform in most samples studied. Whole cow's milk and skimmed milk showed similar levels of free estrone.

Total estrone content of milk was more than one order of magnitude higher than in plasma (Sanchis et al., 1997a). The fact that skimmed milk retained a large proportion of acyl-estrone suggests that, at least initially, esterified estrone was bound to a protein fraction and not dissolved in the fat globules. Manipulation and processing later resulted in a significant migration of acyl-estrone toward the fat moiety of the milk, as, despite a considerable variability in total estrone content, there is a fair overall correlation between the content of fat and that of total estrone ($r = 0.9142$; $P < 0.0001$) in the dairy products studied (Figure 1), a relationship not found when comparing fat content and free estrone ($r = 0.1670$; $P = 0.4976$) levels. In cheeses, the contribution of acyl estrone was 1 to 3 orders of magnitude higher than that of free estrone.

The total content of estrone in most processed dairy products was, however, lower than that corresponding to the crude milk used in its production (i.e., 1 kg of butter requires about 20 L of milk, the estrone content of butter is roughly one-half of that contained in the original volume of milk). Similarly, skimmed milk retains about one-half its original estrone.

The method presented here shows a fairly good degree of recovery of both molecular species measured; however, the recovery from solid materials was not as good as in milk, probably because of adequate accessibility of the solvents used to the core of the fine particles in which the sample was fragmented. The preparation of powdered samples under liquid nitrogen has the added advantage that, at ultra-low temperature, fats are brittle and can be easily ground. Nevertheless, the presence of even tiny amounts of water may prevent a quantitative extraction of acyl-estrone. When dryness was not absolute, recoveries rapidly plunged. High-temperature evaporation of water in an oven gave poor results, probably because of the formation of barrier protein precipitates that retained or occluded part of the acyl-estrone. Powdering, followed by lyophylization, increased the recovery as did the use of ethyl-acetate as solvent for the extraction of estrone from the alkaline medium, as this procedure mini-

mized the carrying of interfering soaps and other lipids.

The integrity of both estrone and acyl-estrone in the dairy products investigated suggests that both molecules are able to withstand both the physical and microbiological manipulations responsible for the production of the products investigated. The fairly uniform free estrone content found in most dairy products investigated may be related to the sparse solubility of this compound in aqueous solutions and/or its binding to milk proteins, resulting in moderate levels because a large part of the free hormone might have been lost with whey or other fractions lost during processing.

Milk contains a high amount of acyl-estrone, along with a significant presence of free estrone; part of the highly lipophilic acyl-estrone of most dairy products studied is loosely associated with higher fat content, but this is not true for whole milk, as semi-skimmed milk and fully skimmed milk retain almost one-half the original acyl-estrone with dwindling fat content. Parmesan cheese made of partially skimmed milk also contained a lower proportion of acyl-estrone than expected. Again, plain yogurt contained much less acyl-estrone than milk, despite having a similar fat content. Thus, the data presented suggest that acyl-estrone is originally carried in the milk mainly bound to protein. Mechanical treatment, microbiological treatment, or both however, resulted in the loss of a large share of total estrone, and most of the remaining acyl-estrone was carried along with lipids, yielding high concentrations in most cheeses.

Whole milk contained a very large concentration of total estrone, in agreement with our previous findings using a less precise method (Remesar et al., 1999). The levels of free estrone in milk are also in a range similar to those described in the literature (Hartman et al., 1998). Levels of total estrone in butter were similar to our previous estimates (Remesar et al., 1999). It must be taken into account, however, that it is possible that the original levels of estrone in milk just produced may be variable, depending on factors such as the age of the sucklings, state of nutrition of the dam, or other environmental or endocrine factors. Consequently, the variability found in several dairy products may be due to this initial variability and not necessarily a consequence of the physical processes, biological processes, or both used in the manufacture of dairy products. Notwithstanding this caveat, the data presented here show that there is a fair degree of uniformity in the estrone content of dairy products.

The amount of estrone carried by milk, in any case, is very high and is well within the range at which its intake may exert a physiological response in the sucklings for which it is provided. These growth-inducing and energy expenditure-lowering effects (Sanchis et al., 1996; Remesar et al., 1999) may, in turn, induce effects—so far not investigated—in humans ingesting significant amounts of dairy products. The growth-inducing effects of milk-based diets for children may well be a consequence of its high estrone content. However, further studies are needed to contrast this hypothesis.

ACKNOWLEDGMENTS

Funding by grants 01/1300 and 01/309 from the Fondo de Investigaciones Sanitarias and AGL2000-0780 from the Programa Nacional de Recursos y Tecnologías Agroalimentarias from the Government of Spain are gratefully acknowledged.

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