Analysis and Stability of Orotic Acid in Milk

B. SAIDI and J. J. WARTHESEN

Department of Food Science and Nutrition
University of Minnesota
1334 Eckles Avenue
St. Paul 55108

ABSTRACT

A method based on HPLC and ion pairing techniques was used to determine the orotic acid concentrations in cows' milk. Average amounts were 69 to 74 mg/L. Orotic acid in milk was stable to heating but decreased during commercial yogurt fermentation. Correlations were made between the loss of orotic acid and the increase or decrease in pH during fermentation.

INTRODUCTION

Orotic acid is an intermediate in pyrimidine biosynthesis (22) and therefore a component of all living cells. It is a growth factor for lactobacilli (26) and other microorganisms (6). Orotic acid also has caused fatty livers in rats (9, 22), inhibited hepatic cholesterol biosynthesis in rats (29), and decreased serum cholesterol in humans (23). Orotic acid concentrations in the milk and urine of dairy cows have been used to detect partial deficiency of uridine monophosphate synthase, which is involved in synthesis of pyrimidine nucleotides (24). Orotic acid content also has been used to determine the amount of milk or milk products in bakery products, ice cream, infant foods, and chocolate (20, 21).

Counotte (7) found that the orotic acid concentration in milk of 2250 Friesian dairy cows in the Netherlands averaged 53.8 mg/L with a standard deviation of 21.1 mg/L. Durschlag et al. (9) reported an average of 81.1 mg/L and a standard deviation of 48.6 mg/L for 250 cows in Illinois. Larson and Hegarty (16) found an average of 75 mg/L in 6 samples of Illinois milk. Laurant and Vignon (17) reported an average of 62.4 mg/L and a standard deviation of 17.6 mg/L in 172 samples of milk from French cattle. Several factors have been implicated as causing these variations observed in orotic acid, including heat treatments of the milk and variation in analytical methods (2, 17, 30, 31). Heat stability of orotic acid in cow's milk during processing could be of practical importance, yet little information has been reported on the effect of heat treatment. In their studies of commercial heat processing of milk, Gil and Sanchez-Medina (11) and Munchberg et al. (19) concluded that the orotic acid content of milk is not greatly influenced by the temperature of heating but rather by the length of heating time. Fermentation had a significant effect on orotic acid as reported by Deeth and Tamime (8) and Arla (4). They reported that yogurt fermentation reduced orotic acid by 43 and 47.8%, respectively. The kinetics of orotic acid loss during fermentation could be of practical importance for the control of yogurt production and yet no work has been reported on the rate of orotic acid loss during fermentation.

Various methods have been used for the determination of orotic acid in milk. These include enzymatic methods (5, 10), colorimetric methods (1, 28), microbiological methods (13, 16), and chromatographic methods. Most chromatographic methods are based on ion-exchange chromatography (14, 18) although reverse-phase chromatography was used by Counotte (7) and by Tiemeyer et al. (27). Because of the importance of orotic acid in human health, the growth requirement of some microorganisms, and other applications, this study was carried out to establish
the reliability of a reverse-phase HPLC method for determination of orotic acid, to evaluate the heat stability of orotic acid in milk, and to determine the kinetics of orotic acid loss during an industrial yogurt fermentation.

**MATERIALS AND METHODS**

**Measurement of Orotic Acid**

The HPLC system contained a Waters model 6000A solvent delivery system, a Rheodyne (Rheodyne, Berkeley, CA) model 7120 injector with a 20-μl sample loop, and a Waters (Waters Associates, Milford, MA) model 440 absorbance detector fitted with either 254 or 280-nm filters. The 280-nm filter was used for most of the orotic acid quantitation. Detector output was recorded on a Hewlett-Packard 3380A recorder integrator (Hewlett-Packard, Avondale, PA). Orotic acid concentration was measured using peak height. Separation by reverse phase was accomplished with an endcapped octyl column (250 × 4.5 mm i.d.) with 5-μ particle size (IBM Instruments, Inc., Wallingford, CT). Mobile phases of various compositions were examined for optimal separation of orotic acid in milk. The mobile phase determined to be optimum for orotic acid measurement consisted of 5% ACS grade methanol (Fisher Scientific, Ltd., St. Louis, MO) and 95% .01 M phosphate buffer (pH = 4.0) containing .005 M tetrabutyl ammonium phosphate (Waters). At a flow of 1.0 ml/min, the elution of orotic acid was completed after 10 min. Orotic acid standard was obtained from the Sigma Chemical Company (St. Louis, MO). Chromatographic standards for peak identification and quantitation were prepared in distilled water.

**Sample Treatments**

An extraction method based on the method of Tsugo et al. (28) was used. Milk samples of 5 ml or yogurt samples of 5 g were transferred into test tubes and one ml of 12% wt/vol TCA solution was added. Samples were mixed on a vortex mixer for several seconds and then centrifuged for 20 min at 800 × g. The supernatant was filtered through a .45-μm membrane filter and used directly for HPLC analysis.

**Heat Treatments**

To determine the heat stability of orotic acid, 5-ml samples of pasteurized skim milk were transferred to glass test tubes (16 × 125 mm). The test tubes were capped and placed in covered water baths at either 80 or 100°C for up to 48 h. Duplicate tubes were sampled at various intervals, and orotic acid levels were measured.

**Yogurt Fermentation**

This study was conducted using a commercial yogurt process in Morocco. The yogurt was produced from a whole milk in which the solids content was increased by addition of NDM. This milk represents pooled milk from commercial processing and would not be from any particular breed of cattle. The milk was then homogenized, pasteurized (96°C, 3 min), cooled to about 45°C, and inoculated with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The milk was placed in commercial-sized packages and incubated in a temperature-controlled room at 45°C.

To determine the kinetics of orotic acid reduction during an industrial fermentation process, duplicate samples were taken from a selected batch at the beginning of the incubation step and then every 15 min until the end of the process. Titratable acidity and pH also were measured (3) in order to follow the fermentation process. To stop the fermentation, yogurt samples were immediately frozen and held at -20°C until analysis. Fat in yogurt was 1.7% and in total solids was 21%.
Statistical Analysis

Linear regression with transformation of the dependent variable (orotic acid retention or percentage acidity) to determine the best fit line was used to determine reaction order of orotic acid loss and acidity increase during fermentation. A linear relationship was compared to a semilogarithmic relationship.

RESULTS AND DISCUSSION

Measurement of Orotic Acid in Milk

In the analysis of milk samples using reverse-phase chromatography, orotic acid elution was fast (about 1 min) when the mobile phase was 100% phosphate buffer without an ion pairing agent. This was a problem since other UV-absorbing compounds in milk eluted with orotic acid. Tiemeyer et al. (27) doubled the column length to increase the retention of orotic acid. An alternative to standard reverse-phase chromatography for the quantification of orotic acid is ion pair chromatography. A number of combinations of methanol concentrations with tetrabutyl ammonium phosphate were evaluated, and the best mobile phase providing resolution of orotic acid from other compounds was used. A chromatogram showing the resolution obtained for orotic acid in a skim milk sample is presented in Figure 1. The mobile phase was 5% methanol, 95% phosphate buffer (pH = 4.0), and .005 M as the final concentration of the ion pairing agent.

The ion pairing mobile phase developed in this study provides some chromatographic flexibility because the amount of methanol can be varied between 5 and 20% when changes in elution time are necessary due to gradual column changes. Detector response at 280 nm was linear in the range of concentrations tested (8 to 80 ppm).

The identity of orotic acid in milk extracts was confirmed by addition of standard orotic acid solutions to the samples and by comparing the peak height ratio at 280 and 254 nm of a standard to that of a sample. This ratio was constant and equal to 1.6 for the orotic acid standard and the peak identified as orotic acid in the sample.

The developed HPLC method was suitable for orotic acid quantification in heated milk and yogurt. Orotic acid concentrations were determined in four retail skim milk samples obtained in St. Paul, MN and three skim milk samples obtained during three different weeks from the University of Minnesota herd. The average concentration was 74 mg/L with a standard deviation of 16 mg/L and a range of 52 to 98 mg/L. Orotic acid concentrations in Moroccan whole milk were also determined using eight retail whole milk samples obtained in Rabat. The average concentration was 69 mg/L with a standard deviation of 8 mg/L and a range of 55 to 80 mg/L. These values show similar concentrations in milk obtained from two distinctly different locations.

To measure the precision of the determination of orotic acid, five milk samples were deproteinized in triplicate, and orotic acid was measured. The coefficient of variation of the concentration of orotic acid due to variations in the analytical procedure was between 0 and 2.7% with an average of 1.7%. The coefficient of variation of the determination of orotic acid by chemical analysis and by bioassay were 6.9 and 12.8%, respectively (15). The average coefficient of variation found by

Figure 1. Chromatogram of orotic acid in a milk extract. Orotic acid elutes at 9.40 min.
Counotte (7) for his HPLC method was 1.29%. Therefore, the determination of orotic acid by HPLC is rapid, sensitive, and precise. The developed method is faster and more flexible than the Counotte method with similar precision.

The orotic acid concentrations in milk found in this study are in agreement with reported values (2, 4, 16, 18, 22). The average values reported by these authors were between 67 and 81 mg/L with large standard deviations. Other authors found somewhat different values. Averages reported by Counotte (7) and Laurant and Vignon (17) were lower, 53.8 and 62.4 mg/L, respectively. Gil and Sanchez (10) reported an increase in orotic acid from 7.6 mg/L after parturition to 65.4 mg/L during different stages of lactation. Differences in orotic acid content can be attributable primarily to cow-to-cow variation and secondarily to the stage of lactation (22).

**Heat Stability of Orotic Acid**

When milk samples were held at 80°C for up to 48 h and at 100°C for up to 19 h, the losses of orotic acid were 8 and 12%, respectively. These losses are not large enough to generate kinetic analysis, and the degradation of orotic acid is probably not significant under normal heat processing conditions. These results agree with the finding of Tsugo et al. (28), who reported that orotic acid is very stable to normal pasteurization treatments. Gil and Sanchez-Medina (11) found that HTST and UHT treatments did not affect orotic acid; however, the sterilization of milk in bottles at 120°C for 20 min in some cases had a significant effect on orotic acid. The calculated values from their data show a large discrepancy in orotic acid loss depending on the method of analysis. These values were 20 and 6.5% when the ion-exchange chromatography method and enzymatic method, respectively, were used. From their results, Gil and Sanchez-Medina (11) and Munchberg et al. (19) concluded that the orotic acid content of milk was not reduced by the heating temperature but by the length of heating time. This conclusion is very limited and may be valid only to the data generated by the authors, since a general conclusion on heat degradation should be based on Arrhenius Law. Our results did not suggest heat sensitivity under practical processing conditions.

**Influence of Yogurt Fermentation on Orotic Acid Content**

Although orotic acid was heat stable, fermentation had a more pronounced effect on orotic acid. Data for orotic acid retention, pH decrease, and acidity increase developed during yogurt fermentation are presented in Figure 2. Lines were determined using linear regression based on zero-order kinetics. The orotic acid reduction and acidity increase were better described (higher r²) by linear relationships when compared to semilogarithmic relationships.

| Table 1. Zero order rate constants (k) and r² values for orotic acid retention, pH decrease, and percentage acidity increase during fermentation. |
|-----------------|-----------------|-----------------|
|                 | k               | r²              |
| Orotic acid retention | -22.40 %/h     | .994            |
| pH Decrease       | -1.17 units/h   | .980            |
| Acidity increase, | 3.44 %/h        | .979            |

Figure 2. Changes in orotic acid level (●), acidity (△), and pH (●) during yogurt fermentation.
Each regression line was calculated using 18 data points (9 sampling times with duplicate samples for each time). Data points shown are means of duplicate samples. Calculated zero-order rate constants (k) and $r^2$ for orotic acid retention, pH decrease, and acidity increase are reported in Table 1. The results for acidity increases and pH decrease follow the same trend as suggested by Greig and Van Kan (12) in their study of the effect of the protein concentrate on yogurt fermentation.

At the end of the incubation process, orotic acid was reduced by 45%. This is in agreement with the data reported by Deeth and Tamime (8) and Arla (4). Arla reported an orotic acid reduction of 47.8%, and the calculated value using Deeth and Tamime data is 43% reduction in orotic acid in yogurt.

The percentage retention of orotic acid as a function of acidity and as a function of pH was compared by linear regression. There was a good correlation between orotic acid retention and acidity ($r^2 = .970$) and between orotic acid retention and pH ($r^2 = .960$). These results suggest that orotic acid retention during the fermentation process can be used like titratable acidity or pH for monitoring yogurt fermentation (25). Although acid or pH is much easier to measure, orotic acid retention may provide additional information about the type or extent of fermentation and can be used to characterize yogurt fermentation.

REFERENCES

