methods

Simplified methodology to determine breast milk retinol concentrations

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Abstract **The impact of a nutritional intervention trial on vitamin A status can be evaluated by measuring the total vitamin A concentration in breast milk both before and after the intervention. Because breast milk contains a spectrum of retinyl esters as well as retinol and high lipid content, determination of total breast milk retinol routinely requires saponification with alcoholic potassium hydroxide. Retinol is then extracted with an organic solvent, usually hexanes, before HPLC analysis. Retinyl acetate, although commonly used as an internal standard, is not ideal because it can only be added after saponification and extraction and consequently, will only account for part of the total losses. A method has now been developed that uses 3,4-didehydroretinyl acetate (DRA) as an internal standard. DRA is an excellent choice as an internal standard for the following reasons: *1*) DRA can be added to the breast milk before saponification and can be carried through the analysis as dehydroretinol (DR), *2*) the percent recovery can be easily determined, and *3*) DR is easily separated from retinol during HPLC analysis. The procedure, as described, typically gives a mean extraction efficiency of 80–90%. Moreover, the average coefficient of variation is <5% on the same sample run several times in the same day.**—Tanumihardjo, S. A., and K. L. Penniston. **Simplified methodology to determine breast milk retinol concentrations.** ***J. Lipid. Res.*** **2002.** 43: **350–355.**

Supplementary key words saponification • dehydroretinol • vitamin A

Vitamin A is an essential nutrient for humans (1). The vitamin A statuses of women and children in both the underdeveloped world (2–4) and the United States (5, 6) are compromised. Multiple reasons for this compromised status are evident. For women, low dietary intakes and the increased demands during pregnancy and lactation are certainly the main reasons for vitamin A inadequacy along with social and environmental factors. For children, inadequate dietary intake and multiple childhood infections can cause children to deplete their vitamin A liver reserves (7). While vitamin A status assessment has advanced greatly in the past decade, a need for simplified, accurate methodology still exists. Improved methods are needed to accurately assess both individuals and populations at risk of either vitamin A deficiency or toxicity. Dietary surveys are usually not enough in determining the vitamin A status of groups because of multiple factors that affect the absorption and utilization of the pro-vitamin A carotenoids and pre-formed vitamin A (8). While clinical signs of deficiency have been used in the past, i.e., Bitot's spots and xerophthalmia, a marginal vitamin A status is much harder to diagnose (9). Moreover, clinicians and nutritionists want to diagnose vitamin A depletion before clinical symptoms occur as a marginal vitamin A status has been linked to increased morbidity and in some cases mortality (7).

Vitamin A status assessment is not a straightforward procedure. Serum concentrations of vitamin A (retinol) are homeostatically controlled and do not begin to decline until liver reserves are dangerously low (10). Also, in times of infection, serum concentrations are transiently reduced due to the acute phase response (11). In a healthy individual, the liver contains approximately 80–90% of the total body reserves of vitamin A mostly in the form of retinyl esters. A liver reserve of ⩾0.070 μmol retinol/g liver has been defined as an adequate amount for humans. The reported range of liver reserves in well-nourished healthy American adults is 0.44–0.74 μmol/g (10). However, liver biopsies of humans are only justifiable in certain instances and therefore indirect methods to determine total body reserves are needed.

Vitamin A assessment methods that will predict the liver reserves of a population with the smallest number of samples and are minimally invasive are still in development. Biochemical assessment techniques that have been used include the relative dose response (RDR) and modified relative dose response (MRDR) tests (12), and the deuterated retinol dilution (DRD) assay (13–15). Breast milk retinol concentrations have also been used as an indicator of vitamin A status of a community (16, 17). Breast milk collection is less invasive and usually easier than blood drawing. Breast milk samples also do not have to be further processed at the field station, thus, shortening sam-

Abbreviations: BHT, butylated hydroxytoluene; CV, coefficient of variation; DR, dehydroretinol; DRD, deuterated retinol dilution; DRA, 3,4-didehydroretinyl acetate; ext. eff., extraction efficiency; MRDR, modified relative dose response; RDR, relative dose response.

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ple preparation time. While a unique indicator to lactating women, the status of the mother can usually be predictive of the nursing infant (17). Therefore, if the lactating women of a community have a marginal vitamin A status, chances are high that the children of that community are also at risk of vitamin A depletion (2, 12, 17). Evaluating the nutritional impact of an intervention trail on vitamin A status can be done by measuring retinol levels in breast milk both before and after the intervention (16, 18).

In this article we describe simplified saponification methodology to assess breast milk retinol concentrations using 3,4-didehydroretinol as an internal standard and HPLC for analysis. We also investigate some issues that may affect success of the methodology application, i.e., uniform sample volume, use of antioxidant, and length of saponification time.

MATERIALS AND METHODS

The collection of breast milk from human subjects was reviewed and approved by the University of Wisconsin-Madison College of Agricultural and Life Sciences Human Subjects Committee. Written informed consent was obtained from the individuals who gave breast milk for the methods development.

3,4-Didehydroretinyl acetate (DRA) was synthesized using published procedures (19, 20). The compound was carefully purified on an 8% water-deactivated alumina column and dissolved directly into 100% corn oil by sonification. An appropriate working dilution was made to obtain approximately 10 μg/μl; the $^{1\%}E_{1cm}$ of 3,4-didehydroretinol is 1455 (21). This oily solution was stored at −80°C. When DRA is dissolved in oil and stored frozen at −80°C, we have found it to be stable for at least 5 years. (Small quantities of DRA are available for breast milk analysis directly from Tanumihardjo at UW-Madison, Department of Nutritional Sciences, for a shipping and handling charge.)

From the DRA stock solution, 10–15 μl (100–150 μg) was mixed with 25 ml isopropanol in a volumetric flask. Forty microliters (160–240 ng) of this solution was added to each aliquot of breast milk to be analyzed. Ten microliters of this solution was injected into the HPLC system and eluted (retention time ~10 min on a 15 cm C-18 column under conditions described below). Dependent upon the column, the *cis-trans* isomers may be separated. The integrator areas of the isomers are added together and this area represents 100% recovery of the dehydroretinol (DR). The detector responses have been shown to be similar for the free alcohol and the ester (22). On reverse-phase columns, the DR isomers most likely will not be separated. The extraction efficiency (ext. eff.) was obtained by dividing the integrator area obtained for the DR by the expected area obtained with the 10 μl dehydroretinyl acetate:

$$\text{Ext. eff.} = \frac{\text{Area obtained for DR}}{\text{Expected area from DRA}}$$

The samples were corrected by dividing the vitamin A value obtained in μmol/l by the ext. eff.:

$$\text{Corrected value} = \frac{\text{Calculated value}}{\text{Ext. eff.}}$$

All analyses were conducted under yellow lights. To ensure complete mixing of breast milk, the breast milk was completely thawed and gently, yet thoroughly, mixed prior to aliquotting samples. Aliquots of 250 μl (n = 6) and 500 μl (n = 6) breast milk from a healthy volunteer (subject L) were pipetted into screw top test tubes. Forty microliters of the internal standard solution were added. The samples were suspended into ethanol (1.5 × volume) by vortexing for 15 s. To this suspension was added 50:50 (W/V) KOH-HOH (0.8 × volume). The samples were vortexed for 15 s and placed in a water bath at 45°C for 30, 60, or 90 min (n = 2 at each volume and time interval). The samples were vortexed every 15 min for about 15 s. After the reaction, the samples were extracted three times with 2× volume of hexanes by vortexing for 30 s and centrifuging for 30 s to aid in separating layers. The top organic layers were pooled into a clean test tube and evaporated under argon. The residue was redissolved in 100 μl of 50:50 methanol-ethylene dichloride and 25 μl was injected onto the HPLC system.

The wavelength of detection of a Shimadzu SPD-10A UV-VIS detector was set at 335 nm, which is mid-way for DR (maximum at 350 nm) and retinol (maximum at 325 nm). The stationary phase was a Phenomenex 15 cm, 5 μm, C-18 reversed-phase column (Torrance, CA). The mobile phase was 89:11 methanol-

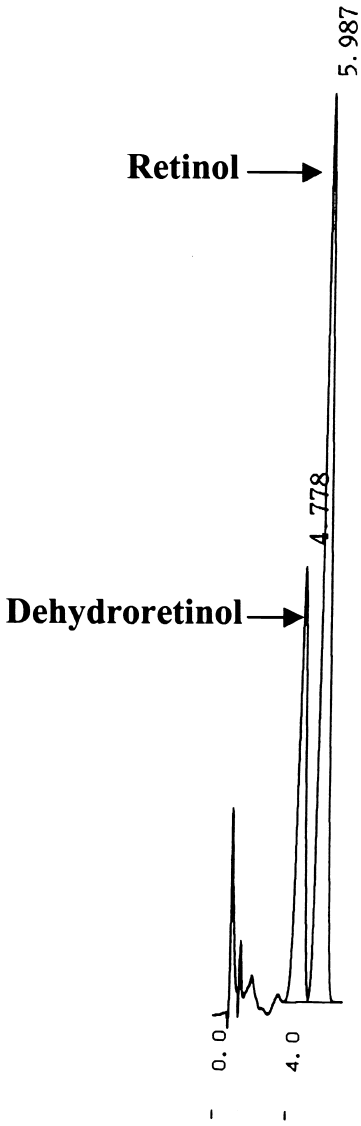

Fig. 1. A HPLC chromatograph of a saponified breast milk sample (subject H) with dehydroretinol (DR) (retention time ~4.8 min) as the internal standard and retinol (retention time ~6.0 min) as the analyte of interest. The eluting solvent was 89:11 methanol and water at a flow rate of 1.2 ml/min on a 15 cm, 5 μm, C-18 reversed-phase column.

water at a flow rate of 1.2 ml/min delivered by a Beckman 110B pump. A Shimadzu C-R7A Chromatopac data processor recorded and calculated peak areas. All standards, both internal and external, were run at the same wavelength as that used for the analysis. External standardization was performed by using HPLC purified retinol prepared by saponification of retinyl acetate.

Breast milk (50 ml) from two other healthy volunteers was obtained (subjects M and H). Each sample was aliquoted (500 μl) into 12 screw top test tubes. The above saponification procedure was followed except butylated hydroxytoluene (BHT) was added as an antioxidant at the rate of 0.1% (W/V) in the ethanol in half of the samples analyzed, that is n = 6 of each subject with and without BHT. The samples were again saponified at 45°C for 30, 60, or 90 min [n = 4 at each time (two with and two without BHT)].

All data were entered into a Microsoft Excel spreadsheet. Averages, standard deviations, coefficients of variance and appropriate *t*-testing were performed on the data. Using SAS Version 8 (Cary, NC), a test for equality of slopes with procedure GLM was performed to determine differences based on saponification time and addition of antioxidant (total n = 36).

RESULTS

Figure 1 illustrates the HPLC profile of 3,4-didehydroretinol and retinol. The retention times for 3,4-didehydroretinol and retinol were 4.8 min and 6.0 min, respectively.

Table 1 outlines the results obtained when different volumes of sample were used from subject L. Even though the percentage recovery varied from 23–89%, dependent on saponification time, the corrected retinol concentration did not change within the analyzed volume group. The coefficient of variation (CV) was 4.1% and 1.8% for the 250 μl and 500 μl samples, respectively. However, the volume of the sample analyzed did make a difference; the 250 μl samples gave a 10% higher value than the 500 μl samples and this difference was significant ($P < 0.01$).

The optimum saponification time for the procedure, which did not include BHT, was 60 min as judged by the percent recovery of the internal standard and the least amount of variation. **Table 2** summarizes the mean concentration of retinol for subjects L, M, and H corrected for internal standard at the different saponification times. Although there was a trend for the 30 min value to be lower than the value obtained at 60 and 90 min, the result within each subject was not significantly lower ($P > 0.10$). However, the coefficient of variation for the retinol concentration at 30 min for subjects M and H (500 μl) was always much higher than that obtained at 60 min and 90 min (**Table 3**). Furthermore, applying a test for equality of slopes at the different saponification times, the slope of the 30 min time across the different concentrations was significantly different than that obtained at 60 min and 90 min ($P = 0.029$).

When applying a test for difference on the addition of BHT across the various times used in the study, the slopes of the BHT added versus no BHT added are identical for the time points used in this study **(Fig. 2)**. The addition of BHT does have a protective effect on the 3,4-didehydroretinol internal standard as judged by the percent recovery ($P = 0.023$) especially when the saponification time was lengthened to 90 min (Fig. 2). However, the coefficient of variation of the method was always <4% for the 500 μl samples at 60 and 90 min with and without the addition of BHT (**Table 4**).

TABLE 2. Performance of the breast milk assay as a function of saponification time[a] and concentration of retinol in the breast milk[b] (n = 4 at each time and concentration)

Time Saponified	Retinol Concentration		
	Low	Medium	High
min	*μmol/l*	*μmol/l*	*μmol/l*
30	1.11 ± 0.04	2.18 ± 0.20	3.06 ± 0.48
60	1.17 ± 0.08	2.34 ± 0.03	3.57 ± 0.21
90	1.19 ± 0.08	2.29 ± 0.05	3.62 ± 0.10

[a] The slope of the line corresponding to a 30 min saponification time is significantly different from that obtained at 60 min and 90 min across the various concentrations ($P = 0.029$).

[b] The values obtained at each concentration level are not significantly different from each other.

TABLE 1. Performance of the breast milk assay as a function of sample volume used (n = 6 at each volume)

Time Saponified	Volume Used				
	250 μl		500 μl		
	% Recovery of Internal Standard	Corrected Concentration	% Recovery of Internal Standard	Corrected Concentration	% Recovery CV at Each Time Studied
min		*μmol/l*		*μmol/l*	%
30	23	1.13	66	1.06	48.5
	29	1.14	60	1.11	
60	89	1.23	81	1.09	4.0
	84	1.23	86	1.11	
90	66	1.27	78	1.12	14.2
	88	1.24	66	1.13	
Means ± SD		1.21 ± 0.05[a]		1.10 ± 0.02[a]	
CV (%)		4.1		1.8	

[a] The value obtained at sample volume 250 μl is significantly higher than that of 500 μl ($P < 0.01$).

TABLE 3. The coefficient of variation (%) of the breast milk assay (500 μl samples) as a function of time and concentration of retinol in the breast milk (n = 4 at each concentration and time point)

Time Saponified	Medium Concentration	High Concentration
min		
30	9.1	16
60	1.4	5.9
90	2.2	2.7

TABLE 4. The coefficient of variation (%) of the breast milk assay (500 μl samples) as a function of addition of butylated hydroxytoluene (BHT) and concentration of retinol for saponification times of 60 and 90 min (n = 4 in each subgroup)

Treatment Applied	Medium Concentration	High Concentration
With BHT	2.0	3.0
Without BHT	2.3	4.0

DISCUSSION

Historically, retinyl acetate has been used as an internal standard for determination of breast milk retinol concentrations (23, 24). However, this must be added after saponification and therefore only accounts for mechanical loss due to evaporation procedures and not losses during saponification and extraction. In the development of this assay, when retinyl acetate was tried as the internal standard, extraction efficiencies of approximately 0% and 100% were frequently obtained. The 0% meant that the internal standard was being hydrolyzed by residual base and co-eluting with the retinol and the 100% meant that we had no losses in the evaporation step. Therefore, we concluded that the addition of retinyl acetate at this stage did not yield very useful information. More recently, β-apo-8′-carotenal-methyl oxime has been used as an internal standard (17) but this requires separate detection at 450 nm, which is not possible on simple single wavelength HPLC systems.

Other investigators have opted to use no internal standard (18, 25, 26) and claim that the recovery was assumed to be 100% because of exhaustive extraction. While during the development of this method we frequently obtained >90% extraction efficiencies on the same sample run several times in the same day, we never achieved an average of 100%. Moreover, when the method was applied at the population level with women of varying degrees of nutritional adequacy, the extraction efficiency varied widely due to the enormous differences in the fat content of the samples with a mean approaching 80% (unpublished observation). We attributed this to either over or under saponification at the selected time of 60 min (an antioxidant was not used in the analysis). Referring to Table 1, the DR corrected for the incompletely or over saponified samples yielding very small CVs in this study. That is, 4.1% for the 250 μl aliquots and 1.8% for the 500 μl aliquots. Moreover, when we look at the difference in the addition of the antioxidant BHT to the reaction mixture, although the average % recovery varied from 70% to 91% at the 60 min and 90 min saponification times, the range in the CV of the method was 2–4% for the same sample run several times (Table 4). It seems that the rate of degradation and solubility of the retinol and DR are similar.

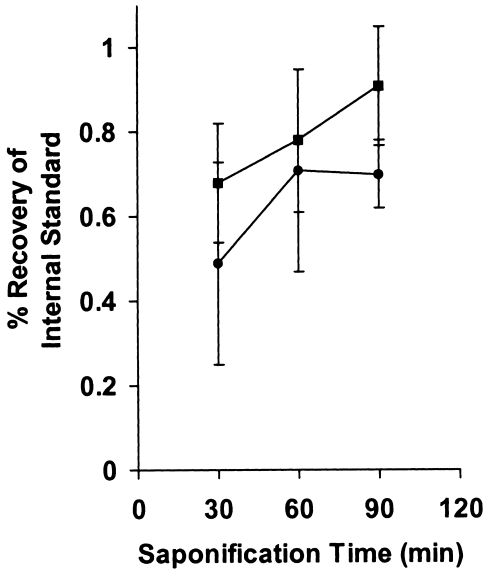

Fig. 2. The percentage recovery of the 3,4-didehydroretinol internal standard as a function of saponification time in the presence (closed square) and absence (closed circle) of butylated hydroxytoluene (BHT). The slopes of the lines do not differ; however, the BHT does yield a significantly higher percent recovery of the internal standard ($P = 0.023$).

Ideally, precise aliquots to be used in the analysis should be removed and placed in separate vials under an inert atmosphere for long-term storage soon after collection. Once breast milk is frozen and thawed it is difficult to get complete homogenization. For example, with our subject H who had a correspondingly high retinol concentration, by not fully thawing and mixing, the value obtained was significantly higher (unpublished observation), most likely due to thawing of the lipid layer before the water layer. Especially under field conditions, where the person collecting the breast milk is usually not the same as the person doing the analysis, it is important to thoroughly thaw and mix the breast milk before aliquotting the samples. Otherwise, duplicate retinol values from the same individual may be quite different due to inconsistent fat content. When expressing the vitamin A content by volume, the collection period is better if it is randomly distributed throughout the day to account for fat variation in the milk (9). If this is not possible, one may want to account for the fat content by published procedures (27–29).

Several factors contribute to the success of analytical methods. Having small CVs for the same subject is important especially when the concentration of the analyte of interest, e.g., breast milk retinol, varies widely among individuals. This point is even more important when breast milk retinol concentrations are used in the evaluation of an

intervention trial. All of our subjects were well-nourished; however, there was a threefold difference in the retinol concentration that was not planned. Regardless of the retinol concentration, the CV was always ⩽3% at conditions that we found optimal (i.e., standard volume of 500 μl, addition of BHT and saponification times of 60 min and 90 min). Although the DR was able to correct for incomplete or over saponification, having high percentage recoveries in an analytical method is also desirable. We observed average extraction efficiencies of 78% and 91% for 60 min and 90 min saponification times, respectively. The lower value at 60 min was most likely due to incomplete saponification of samples from subject H, which made it harder to redissolve the residue before injection into the HPLC. However, the DR corrected for this discrepancy. Moreover, with samples from subject L, which did not include BHT, even though the percent recoveries declined after 60 min (Table 1) due to over saponification and hence degradation, again the DR corrected the value.

Another advantage to the method described is that the HPLC run time is quite short (<7 min, Fig. 1) and the solvents used are quite common and inexpensive globally. Moreover as the samples are saponified, we had no problem doing an entire day's worth of samples before washing the non-polar breast milk constituents off from the reversed-phase column with methanol-dichloromethane (50:50). These points are important when faced with the large number of samples needed for population assessment of vitamin A status using breast milk retinol concentrations.

DRA is an excellent choice for an internal standard for the following reasons: *1*) DRA can be added to the breast milk before the analysis is started and can be carried through until the HPLC determination, *2*) DRA is in the esterified form and will therefore undergo hydrolysis as do the retinyl esters in the breast milk, and *3*) dehydroretinol is easily separated from retinol during the HPLC analysis. Even though the fat content of breast milk varies widely within a population, thereby affecting the degree of saponification for individual samples in a uniform procedure, the DR effectively accounts for this difference.

Therefore, in the application of the breast milk retinol assay to population studies, we make the following recommendations based on this study: *1*) a uniform sample volume should be used for the analysis of all subjects (i.e., 500 μl), preferentially aliquoted prior to freezing; *2*) an appropriate saponification time (i.e., 60 min) should be used; *3*) an antioxidant (i.e., BHT) is recommended when saponification times are not uniform, and *4*) using internal standards, like DR, will improve the CV of the analysis.

This work was funded in part by Hatch-Wisconsin Agricultural Experiment Station number WIS0438 and the UW-Graduate School. The authors also thank Peter Crump, Senior Information Processing Consultant at UW-Madison, for his assistance in the statistical analysis of the data.

Manuscript received 13 August 2001 and in revised form 6 November 2001.

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