Fluorometric Determination of Vitamin A in Dairy Products

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Abstract

A rapid method has been devised for the determination of vitamin A (retinol) in milk, artificially fortified milk, butter, cheese and ice cream. Samples (1 ml or 1 g) were saponified and extracted with petroleum ether in stoppered centrifuge tubes. Retinol content of extracts was measured fluorometrically. Chromatography of the unsaponifiable lipids on columns of alumina and hydroxyalkoxypropyl Sephadex demonstrated that retinol was responsible for almost all of the measured fluorescence. Retinol amounts are reported for Canadian whole milk, 2% fat-milk and fortified 2% fat-milk. Samples were obtained monthly between March and November 1971. Mean retinol (μg/100 ml±se) in whole milk was low in March (28.3±3.0) and November (32.1±1.5) and maximal in September (45.8±2.3). A similar cycle was observed in the values for 2% fat-milk which invariably were approximately two-thirds those of whole milk. Sixteen out of 18 fortified milks met their label claims but two had only 19 and 22 μg retinol/100 ml.

Introduction

Dairy products are important sources of vitamin A (1). The vitamin A content of milk however varies with the seasons (2) and it is markedly reduced when milk is skimmed. As skimmed milk is sometimes artificially fortified with vitamin A a need has arisen for an analytical method to permit routine monitoring.

Analytical methods used hitherto for the estimation of retinol in milk have had to contend with interference from various classes of lipids including carotenoids. Usually lipids have been extracted, saponified, and chromatographed and then retinol has been measured spectrophotometrically or colorimetrically (3, 4). Recently, it was found that retinol could be measured fluorometrically in hexane extracts of blood serum and in unsaponifiable lipids from liver providing that interference from a fluorescent carotenoid, phytofluene, was eliminated (5). However, in preliminary investigations phytofluene was not detected in milk and thus a simple direct fluorometric determination of vitamin A appeared feasible. Details of such a method and tests of its validity are described, together with results of its application to various types of milk sold in Canada. The method was also applied to other dairy products.

Procedures

Instrumentation, solvents and reagents. Absorption spectra were recorded with a Beckman DB-C spectrophotometer. Fluorescence was measured with a Hitachi Perkin Elmer MPF3 fluorescence spectrophotometer. Hexane (glass distilled, Burdick and Jackson Laboratories, Inc) and ethyl acetate (spectroquality, Matheson, Coleman and Bell) were used as obtained. Petroleum ether (bp 30 to 60, Mallinckrodt analytical grade) and benzene (toxicographic grade, Anachemia Chemicals) were distilled. Ethanol was distilled from KOH pellets. Alumina was washed as described (5) and hydroxyalkoxypropyl Sephadex (HAPS) was prepared from Sephadex G-25 superfine and Nedo 1114 (6). Retinyl acetate was from USP vitamin A reference capsules.

Calibration of fluorescence spectrophotometer. Although retinol standards can be assayed with each set of samples this is tedious in practice. It was more convenient in the present method to calibrate the instrument less frequently and make day-to-day adjustments of the sensitivity with a standard solution of quinine sulphate as a reference. It is emphasized that retinol is unstable and that the accuracy of fluorometric and other determinations can be seriously impaired if standards are not carefully prepared.

The method used to calibrate the MPF3 was as follows. The contents of a capsule of retinyl acetate concentrate were squeezed into a 15 ml centrifuge tube and heated for 20 min in a boiling water bath with 1 ml water and .5 g

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KOH. Water (1 ml) and hexane (5 ml) were added and the contents shaken.

An aliquot (2 ml) of the hexane layer was chromatographed on a 1 cm diameter column of 5 g alumina weakened with .2 ml water. The column was eluted successively with 50 ml portions of petroleum ether, 10% diethyl ether in petroleum ether and 50% diethyl ether in petroleum ether. The position of retinol on the column was followed from the fluorescence elicited with a manual ultraviolet lamp. Purified retinol was eluted in the last fraction and evaporated to .5 ml in a stream of nitrogen. This solution was diluted in hexane until the absorbance at 325 m\(\mu\) was approximately .2. The concentration was calculated using a coefficient \(E = 1 \text{ cm}^{-1} \text{% of } 1,830\), and diluting the solution twentyfold to produce a standard of known concentration containing approximately .05 \(\mu g\) retinol/ml. The fluorometer was set at 330 m\(\mu\) excitation and 480 m\(\mu\) emission with both slits at 8 m\(\mu\) band pass. The retinol solution was inserted and the sensitivity was adjusted until 50 scale units corresponded to .05 \(\mu g\) retinol/ml. Hexane was then inserted and a blank reading (1 unit) was measured with the same settings. The retinol solution was replaced and the sensitivity was readjusted so that .05 \(\mu g\) retinol/ml corresponded to 50 plus the reading of the hexane blank. Without further adjustment, a standard solution of quinine sulphate (.01 \(\mu g\) quinine/ml) in .1N \(H_2SO_4\) was read in the instrument. In subsequent analysis the instrument sensitivity controls were set so that the quinine solution gave the same reading as when the instrument was calibrated.

The fluorescence of retinol is linearly related to concentration until self-absorption becomes significant (.25 \(\mu g/ml\)). However, it is cautioned that in some fluorometers, the factors between coarse settings change and are often not precisely as indicated on the dial. Therefore the coarse sensitivity switch should be calibrated or, if possible as with the MPF3, adjusted before linearity is assumed over the full range of the instrument.

Examination of milk lipids. Aliquots of milk (1 ml) were homogenized with 1 ml ethanol and 10 ml petroleum ether. Fluorescence measurements were made on the separated petroleum ether extracts.

Analysis of vitamin A in milk (recommended procedure). Milk (1 ml) was saponified in a 15 ml stoppered centrifuge tube by adding 5 pellets (.5 g) KOH and 2 ml 1% ethanolic pyrogallol and heating for 20 min in a bath of boiling water. Water (2 ml) was added and the digest was extracted twice with 5 ml portions of petroleum ether. The extracts, which were removed after centrifugation, were combined and their total volume was adjusted to 10 ml. The fluorescence of this solution was measured at 330 m\(\mu\) excitation and 480 m\(\mu\) emission with both slits set at 8 m\(\mu\) band pass. Blank trials were made with water instead of milk. The blank reading was subtracted from the sample readings and using the calibration procedure described, the retinol content of the milk was given directly in \(\mu g/100\) ml.

Analyses of vitamin A in butter, cheese, and ice cream (recommended procedure). Butter (1 g) was shaken with hexane (5 ml) and centrifuged. An aliquot of the clear supernatant (1 ml) was evaporated in a tared flask and the fat was weighed. A second aliquot (1 ml) was evaporated under nitrogen in a 15 ml stoppered centrifuge tube. Water (1 ml) was added and the lipid was saponified and analyzed according to the protocol for milk, modified in that the digest was extracted with four portions of petroleum ether which were combined and evaporated under nitrogen to 10 ml. Portions of cheese and ice cream were analyzed similarly.

Tests applied to the method. The efficiency of extraction of retinol from the saponification digest was examined by extracting test samples six times with 10 ml portions of petroleum ether. The fluorescence of each extract was measured.

Specificity of the determination was verified by chromatography of the saponified lipids from 1 ml and 5 ml of milk on columns of alumina (5). Four fractions were collected (petroleum ether, 2, 10 and 50% diethyl ether in petroleum ether) and on each the excitation spectrum of the 480 m\(\mu\) fluorescence emission was recorded.

Specificity of the method was also tested by chromatographing saponified lipids from milk (1 ml), butter (.2 g), cheese (.1 g) and ice cream (.5 g) on a column of HAPS. Details of the chromatographic system for analysis of tocopherols will be published elsewhere (7). In this procedure a 25 cm x .5 cm diameter column of HAPS was eluted continuously with a mixture of ethyl acetate, benzene and hexane (3:30:67) and the effluent was passed through a flow cell in an Aminco Bowman spectrophotofluorometer. The fluorescence emission at 475 m\(\mu\) with 320 m\(\mu\) excitation was monitored with a Beckman 25.4 cm recorder set at a slow chart speed (2.54 mm per min).
To test the procedure, milk samples were purchased for analysis once a month for nine consecutive months in Halifax, Montreal, Toronto, Winnipeg and Vancouver. Two to four samples of both whole and 2% fat milk were obtained from each city on each occasion. Fortified 2% fat milk was also purchased in Halifax (17 samples) and Montreal (3 samples). A sample of 2% fat milk was fortified in our laboratory with a commercially available dispersible preparation of vitamin A (Palma-Sperse Type 250A, Hoffmann-La-Roche Ltd.) and serial dilutions in the same milk were prepared for analysis. Fresh milk was also obtained in September from Guernsey cows which had been grazing during the summer.

Results and Discussion

It was not possible to estimate vitamin A fluorometrically in crude lipid extracts of milk. In these extracts the fluorescence of retinol was completely masked by relatively intense fluorescence from an unidentified substance. The latter had maximum emission at 415 \( \text{m} \mu \) and its excitation spectrum had peaks at 290, 302 and 316 \( \text{m} \mu \) (Fig. 1). This interfering substance was removed by saponification of the lipids. Its other properties have not been investigated.

The unsaponifiable lipids from milk, cheese, ice cream, and butter had fluorescence excitation and emission spectra similar in shape to those of retinol (Fig. 1). Chromatography confirmed that retinol was responsible for most of this fluorescence. Thus fluorescence was recovered in only the 50% diethyl ether in petroleum ether fraction when unsaponifiable lipids from milk were chromatographed on alumina: this is the fraction in which retinol is normally found (5). Supporting evidence that retinol was the dominant fluorescent substance in the unsaponifiable lipids was obtained by chromatography on HAPS. The latter is more suitable for identifying sources of fluorescence than conventional chromatography materials such as alumina because it can be thoroughly washed and it is inert: thus it neither generates nor releases fluorescent artifacts. The solvent system chosen for the tests eluted phytofluene after 10 min, retinyl esters after 13 min and retinol after approximately 40 minutes (Fig. 2). When unsaponifiable lipids from milk, butter and cheese were chromatographed, with the fluorometer monochrometers set at 330 \( \text{m} \mu \) excitation and 480 \( \text{m} \mu \) emission, a single peak was obtained after 40 min. Otherwise, the charts had only barely detectable ripples, most of which occurred during the first 20 min of the chromatography (Fig. 2). There was no evidence of significant amounts of phytofluene in milk. It was not even detected in summer Guernsey milk which is especially rich in carotenoids (1).

When saponified milk samples were extracted repeatedly with petroleum ether, only the first and second extracts contained fluorescence typical of retinol. However, with other dairy products, such as butter, fluorescence was
measurable in the first four extracts. Therefore in the recommended procedures, two extracts were combined in the analysis of milk, whereas

Table 1. Recovery of retinol from 2% fat-milk fortified with vitamin A.

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<tr>
<th>Retinol added* (µg/100 ml)</th>
<th>Total retinol found (µg/100 ml)</th>
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<tr>
<td>0</td>
<td>32</td>
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<tr>
<td>30</td>
<td>62</td>
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<td>120</td>
<td>152</td>
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<td>150</td>
<td>185</td>
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*Added as retinyl palmitate, Palma-Sperse Type 250-S, Hoffmann-LaRoche Ltd.

four extracts were pooled in the analysis of other dairy products.

Recovery of vitamin A added to 2% milk was essentially quantitative (Table 1).

The results of the monthly analyses of milk samples are in Figure 3. On each occasion the samples from different cities proved similar, and therefore the results have been combined. The values are similar to those found previously in other countries in that a seasonal cycle is clearly evident (1, 2). Whole milk at all times contained approximately one and one half times as much retinol as 2% fat-milk. Most of the artificially fortified 2% fat-milks tested (18 samples) met label claims. These samples contained 72 ± 9.3 µg retinol/100 ml (mean ± sp) which ranks them as a better source of vitamin A than fresh whole milk. However, two samples were discovered with 19 and 22 µg/100 ml which indicates that they had not been properly fortified.

The fluorometric method does not measure the carotenoid provitamins. However it is envisaged that most routine monitoring will be applied to fortified skimmed milks in which the carotenoids make a relatively small contribution to the total vitamin A activity.

Acknowledgments

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References


