Measurement of vitamin A in milk and cheese by means of high pressure liquid chromatography (HPLC)

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Summary. A high pressure liquid chromatography (HPLC) procedure has been developed for the determination of vitamin A in milk and cheese. Recovery studies and reproducibility data showed an average recovery of 93 ± 6% and a SD of 4%. We also report results obtained from HPLC in comparison with the colorimetric procedure.

Colorimetry is the conventional method for the determination of vitamin A in food products[2]. After saponification of the sample, vitamin A is extracted into petroleum ether, the extract is purified on an aluminium oxide column, then vitamin A is reacted with antimony trichloride generating the characteristic blue color. A maximum of 6 analyses daily can normally be carried out and the quantity of laboratory material used is quite substantial.

TLC and densimetric methods have also been used for assaying vitamins[3], the results obtained with these methods, however, are not very reproducible for the determination of vitamin A[4].

The method we describe here permits the determination of 30 samples per day, and the glassware used could be reduced by one third. The results are reproducible with a relative SD of ± 4%.

High pressure liquid chromatography (HPLC) has been successfully utilized for the measurement of vitamin A in pharmaceuticals[5,6]. It is still seldom applied to food products which are not enriched with vitamin A. Descriptions of this technique[7,8] usually do not include much information on the reproducibility, yield and comparison with the convential method of Carr-Price[2]. Our description of the HPLC method for measuring vitamin A is accompanied by the results of the extraction yield and also compares them with the results obtained by the Carr-Price[2] procedure.

Experimental part. Reagents. The reagents were petroleum ether (40–60 °C), pyrogallol, anhydrous sodium sulphate and potassium hydroxide of the Merck analysis grade (Darmstadt, FRG) and HPLC acetonitrile (Mallinckrodt, St-Louis, Missouri, USA).

Standard solutions. 5 mg retinol (Fluka, Buchs, Switzerland) were dissolved in 100 ml acetonitrile (solution A). Solution B was obtained by diluting 12 ml of solution A to 100 ml with acetonitrile giving a final concentration of 60 ng/10 µl. The standard solutions should be freshly prepared before each analysis because retinol polymerizes in acetonitrile and forms an insoluble product.

Liquid chromatograph. The apparatus used was a Hewlett-Packard 1084B equipped with a Hewlett-Packard detector with variable wavelengths as well as a Hewlett Packard stainless steel column 25 × 0.46 cm filled with Nucleosil $10C_{18}$ (Macherey-Nagel). The solvents for liquid chromatography and the samples for the analysis were filtered before use through a 0.45 µm Millipore filter.The working conditions are shown in table 1.

Procedure. 9 g potassium hydroxide and 60 ml ethanol were added to a brown 250 ml bulb with 10 g cheese or 40 ml milk containing about 20 to 40 µg vitamin A. The mixture was refluxed in a water bath at 60 °C under nitrogen for 30 min and then the bulb was cooled in an ice bath. The content was decanted into a brown glass dropping funnel. The bulb and the reflux tube were rinsed with 25 ml water in several fractions. Vitamin A was extracted from the aqueous phase with 50 ml petroleum ether (5 times). The organic extract was washed with portions of 50 ml water until there was a negative reaction with phenolphthalein. Then sodium chloride was added in order to avoid the formation of an emulsion. The solution was filtered through an anhydrous sodium sulphate layer and the filter was rinsed with petroleum ether. The sodium was evaporated under vacuum at 50 °C with a rotary evaporator. The residue was dissolved in 4 ml acetonitrile and 10 µl of this solution were applied to the column.

Vitamin A was identified by comparing the retention time with that of the standard. The measurement either of the height or of the surface of the standard and sample peaks allowed us to calculate the vitamin A content. Each measurement was repeated at least 3 times with a SD of less than 4%.

Results and discussion. The vitamin A contents of the cheeses and milks analyzj d varied from 200 to 400 µg/100 g and from 30 to 100 µg/100 g, respectively. After saponification the total vitamin occurred in the form of retinol. We have verified the absence of retinoic acid and retinal by comparing the retention times with those of the standard products. Vitamin A was destroyed rapidly under the influence of light (figure 2). The detection limit was 20 ng retinol.

We have noted a linear response of the UV-detector at a

Table 1. Chromatograph working conditions for measuring vitamin A in milk and cheese

Column	RP-18, 10 µm, 25 × 0.46 cm
Mobile phase	Acetonitrile: water 95:5 v/v
Flow rate	2 ml/min
Pressure	77 bar
Temperature	40 °C
Sample	Concentration: 50–90 ng, quantity injected: 10 µl
UV detector	$\lambda = 328$ nm, 0.0032 D.O.
Paper speed	0.5 cm/min

Table 2. Extraction yield of vitamin A in cheese

Initial concentration (µg/100 g)	Quantity added (µg/100 g)	Quantity detected (µg/100 g)	Yield (µg/100 g)	Yield (%)
353	279	650	265	95
353	223	572	219	98
353	315	664	311	99
385	390	681	328	84
417	223	625	208	93
				av 93 ± 6

Table 3. Comparison of the reverse phase HPLC method with the Carr-Price method

Sample	HPLC (µg/100 g)	Carr-Price (µg/100 g)
Milk	64.7	71.6
	83.5	94.4
	61.7	54.4
	61.4	61.0
	57.4	66.7
Cheese	246.8	280.5
	296.7	270.6
	205.9	203.9
	176.5	186.4
	289.4	319.4
	$\Sigma\bar{x}$: 154.4	$\Sigma\bar{x}$: 160.9

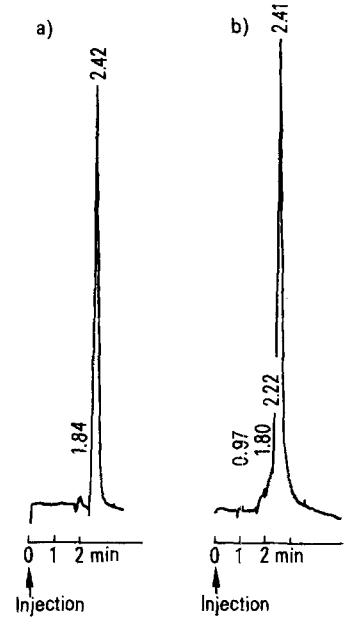

Fig. 1. *a* Vitamin A retinol standard chromatogram. *b* Vitamin A chromatogram from cheese extract.

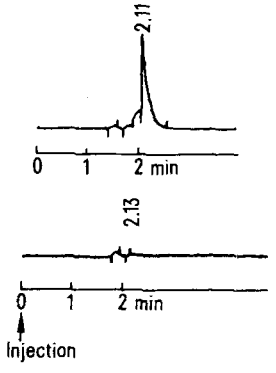

Fig. 2. Vitamin A after a 2-h exposure to daylight.

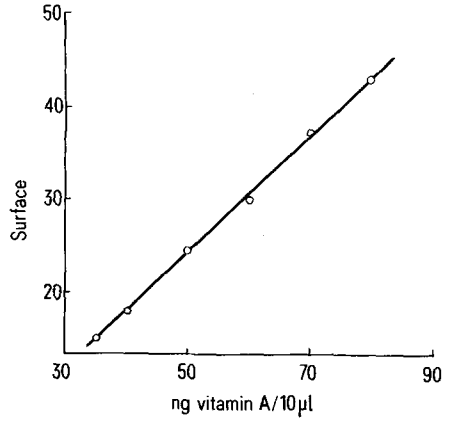

Fig. 3. Linearity of vitamin A content with surface peak area measured with with an UV-photometer.

wavelength of 328 nm between the concentrations of 20 and 120 ng retinol (regression coefficient $r = 0.997$) (figure 3). The necessary quantity was 10 g cheese or 40 ml milk. The measurement of the extraction yield was carried out by adding a known quantity of retinol to cheese before saponification (table 2). The yield is $93 \pm 6\%$.

The results of the measurements using the external standard were reproducible within a limit of less than 4%. A fresh standard was prepared for each test series in order to avoid polymerization of the retinol.

The accuracy of the results was demonstrated by measuring vitamin A in the same sample on different days over a period of 1 month. The factors that might have modified the vitamin A content were eliminated. Each measurement was repeated at least 3 times. The standard deviation was 6% in the sample and 3% in the standard.

We have compared the conventional method (Carr-Price reaction) with the HPLC method for milk and cheese samples. There is no statistically significant difference between the 2 types of method (table 3).

After 20 to 30 analyses, it was necessary to recondition the column when the retention time and the pressure exceeded 5% and 40%, respectively. Reconditioning consisted of a column rinse with an acetone solution (H_2O 95:5) for 1 h. We made about 400 analyses before replacing the column. Reverse phase HPLC is applicable to vitamin A retinol.

In conclusion, we have found that the HPLC method is an efficient technique for measuring vitamin A in dairy products that are not enriched with vitamin A. This method is relatively simple, specific, reproducible and rapid. Due to its rapidity, it is particularly interesting for control analyses. It may also be used for complex samples such as tissues, organs or food products. It offers undoubtable advantages over the conventional procedure.

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