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Determination of Retinol by Aqueous Reverse Phase Open Column System

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DETERMINATION OF RETINOL BY AQUEOUS
REVERSE PHASE OPEN COLUMN SYSTEM

BY

ABDULALY B. AL-ABDULALY

A DISSERTATION SUBMITTED IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY
IN
FOOD SCIENCE AND NUTRITION

UNIVERSITY OF RHODE ISLAND

1986

The ADAC method was first used in the 1950s and was developed by the ADAC method. The ADAC method is a method of measuring the amount of oxygen in a sample. The ADAC method is a method of measuring the amount of oxygen in a sample. The ADAC method is a method of measuring the amount of oxygen in a sample.

DOCTOR OF PHILOSOPHY DISSERTATION

OF

ABDULALY B. AL-ABDULALY

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1986

THESIS ABSTRACT

The AOAC method and other open column techniques which are used to determine retinol content, are time consuming, require technical skills, and do not always result in a complete separation of retinol from other fluorescent components. In some cases cis-trans isomers are formed due to the long term exposure of retinol to oxygen, light, adsorbents and solvents. The colorimetric method has disadvantages which include a rapidly fading blue color, sensitivity of the reagent to moisture, and interference from carotenoids. The more recently developed HPLC method is rapid, reproducible, nondestructive, quantitative and gives high resolution of closely related compounds. The instrumentation, however, is expensive to purchase and maintain, uses expensive solvents and is not available to nutritional scientists in many parts of the world. Therefore there is clearly a need for a method that has the simplicity of the AOAC method coupled with the accuracy and speed of the HPLC method for the determination of retinol in food products and serum.

It was found that packing the HPLC adsorbent (50 μ m C₁₈) in an open column and eluting it with an isocratic, aqueous solvent system consisting of methanol and water as the mobile phase gave good separation of retinol from the extracted sample.

The method was evaluated by measuring the retinol concentration in milk, infant formula, margarine, egg yolk and liver and was compared with both AOAC and HPLC methods. The method was used to measure the retinol concentration in serum as compared to the HPLC method.

The retinol values of milk, infant formula, egg yolk, margarine and liver obtained by RP-C₁₈ open column were very similar to that of AOAC and HPLC methods, and there was no significant differences among these methods when compared over a set of samples. A correlation coefficient between RP-C₁₈ and HPLC estimates and RP-C₁₈ and AOAC estimates were 0.993 and 0.999, respectively, indicating that a highly significant correlation exists between these methods for the determination of retinol from the different samples. The study showed that the RP-C₁₈ method is comparable with both the HPLC and the AOAC methods for retinol analysis from these food products. The recovery study for retinol from the analyzed samples was found to be 97%, 98% and 96% for RP-C₁₈, HPLC and AOAC respectively, indicating that a good recovery was obtained by this method.

Also the study showed that the retinol values of serum obtained by RP-C₁₈ method were very similar to that of HPLC and indicated no significant differences among the

two methods when compared over a different set of samples. A correlation coefficient between the RP-C₁₈ and HPLC estimates was 0.963 for retinol analysis, indicating a highly significant correlation between these two methods. Also the study showed that the RP-C₁₈ method gave comparable values with the HPLC method for retinol from serum.

The retinol separation from the extracted sample could be achieved in 10-15 minutes by the reverse phase open column method. The low cost and ease of operation make this method suitable for routine assays of retinol content in serum and food products. This method can be an alternative technique for clinical laboratories that cannot afford to have HPLC and to nutritional scientists in many parts of the world.

ACKNOWLEDGEMENTS

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PREFACE

This thesis has been prepared in manuscript form according to the style requirements of the Journal of Food Science. The text consists of three manuscripts:

- I. Reverse Phase open column system for the determination of retinol in milk and infant formula.
- II. Reverse Phase open column System for the determination of retinol in margarine, egg yolk and liver.
- III. The determination of serum retinol by reverse phase open column chromatography.

There are two appendices:

- A. Literature review
- B. Bibliography

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ABSTRACT

Abstract text describing the manuscript content, including the title and author information.

MANUSCRIPT I

REVERSE PHASE OPEN COLUMN SYSTEM FOR THE

DETERMINATION OF RETINOL IN MILK AND

INFANT FORMULA

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ABSTRACT

Colorimetric and fluorometric methods are most commonly used for vitamin A analysis. The colorimetric method has disadvantages which include rapidly fading blue color, sensitivity of reagent to moisture and cost of the reagent. The recognized fluorometric method, the AOAC procedure, employs column chromatography before the fluorometric determination to improve sensitivity. This method is time consuming and requires technical skill. The more recently developed HPLC method is rapid, reproducible and quantitative, but the instrument is expensive and requires skilled operators.

It was found that packing the HPLC adsorbent (50 μ m C₁₈) in the open column and eluting it with isocratic, aqueous solvent system (methanol/water) as the mobile phase gave good separation of retinol from the extracted sample. Retinol values of milk and infant formula obtained by this method were very similar to that of AOAC and HPLC methods. Retinol correlation coefficient between RP-C₁₈ and HPLC estimates and RP-C₁₈ with AOAC estimates were 0.988 and 0.999 respectively.

INTRODUCTION

Vitamin A is found in animal products such as milk. It is generally added to milk that is partially or wholly defatted and to infant formulas. It is added in specified amounts to these products usually as retinyl palmitate.

Several methods for vitamin A determination in milk and infant formulas have been published. Colorimetric methods¹ in which vitamin A reacts with antimony trichloride in chloroform (Carr and Price, 1926) or with trifluoroacetic acid (Neeld and Pearson, 1963) to give blue color have been used for both qualitative and quantitative determinations. Several disadvantages associated with these methods such as the instability of the blue color, and sensitivity of the reagent to moisture have been described. A fluorometric method based on the molecular fluorescence properties of the retinol structure (Thompson et al., 1971, 1978) has been used more recently. The method for determining vitamin A in margarine recognized by the AOAC calls for fluorometric determination following alumina column chromatography.

The unsaponifiable portion of the sample is chromatographed on an adsorption column consisting of 2 segments of activated and standardized alumina separated by a middle segment of alkaline alumina. The top segment

of the alumina column prevents caking and initiates separation of vitamin A from carotene and interfering substances. The middle section of alkaline alumina separates persistent interference that cannot be separated by other adsorbents. The final portion of the column is nonfluorescent and provides a suitable background for observing vitamin A fluorescence on alumina.

This method does not always result in a complete separation of vitamin A from other fluorescent components. It is time consuming and in some cases cis-trans isomers are formed due to the long-term exposure of vitamin A to oxygen, light, adsorbents and solvents (Targan et al. 1969, Thompson and Maxwell 1977).

The recent applications of HPLC to the analysis of biological materials has provided the nutritional biochemist with highly useful techniques with a wide variety of applications. The advantages of HPLC over other methods includes its speed, high resolution of closely related compounds and a good recovery of vitamin A in foods (Van DeWeerdof et al. 1973, Frolik and Olson, 1984). The disadvantages of HPLC are that it is expensive to purchase and maintain, and is not available to nutritional scientists in many parts of the world.

Shu-Whei Tsai (1986) reported that packing the HPLC adsorbent (C₁₈) in an open column and eluting the sample

with an isocratic, nonaqueous solvent system (Acetonitrile/methanol/chloroform) as the mobile phase gave good separation of α - and β -carotene from β -cryptoxanthin and lycopene, and the results were the same as that of AOAC method for carrots, and very similar to that of the HPLC method for spinach and peaches.

There is clearly a need for a method that has the simplicity of the AOAC method coupled with the accuracy and speed of the HPLC methods for the determination of retinol in milk and infant formula.

In this study, the concept of aqueous reverse-phase chromatography on highly retentive packing materials was applied in the open column, packed with 50 μ m C_{18} particles as adsorbents for analysis of retinol in milk and infant formula. An aqueous, isocratic solvent system was used as the mobile phase. Nitrogen gas was used to pressurize the column (10 psi) and a conventional spectrophotometer was used for quantification. The comparison of the analysis and recovery for vitamin A by reverse phase open column system, HPLC and AOAC was done for milk and infant formula.

MATERIALS and METHODS

Extraction

Whole milk, low fat milk, skim milk and infant formula were purchased from local supermarkets.

Milk and infant formula were saponified directly with a solution of a 15% KOH in methanol. The alkaline mixture was heated in water bath at 70°C for 30 minutes with occasional mixing. The solution was cooled and transferred to a 250 ml separatory funnel. Ethyl ether (35 ml) was added and shaken vigorously for 5 min. Petroleum ether (5 ml) was added to the funnel, and layers were allowed to separate. The aqueous layer was drawn off and extracted two times with ethyl ether and petroleum ether. The organic solutions were then combined in a separatory funnel and washed free from alkali by repeated addition of water. The resultant aqueous layer was discarded. The organic phase was filtered through Na₂SO₄ to remove moisture and evaporated in a rotary evaporator, the sample was dissolved in a small amount of petroleum ether and stored under nitrogen gas in small vial, at -20°C until analysis (Mills 1985).

Chromatography

Reverse Phase Open Column Procedure

A glass column (160 mm * 11.5 mm) fitted with a sintered disc at the bottom and a 100 ml reservoir at the top (Knotes Glass Co. K-420000) was filled with 6 cm of the 50 μ m C_{18} particles. (Separation Technology, Wakefield, RI). The top surface of the adsorbent was covered with a piece of glass wool. Nitrogen gas from a cylinder was used to pressurize the column (10 psi). (Figure 1) The column was prewetted with the eluting solvent which consisted of a mixture of distilled water and methanol (10:90). The extracted sample was evaporated and then dissolved in the eluting solvent and transferred to the column. Vitamin A was eluted with 100 ml of the mobile phase. The solution was transferred to a separatory funnel containing 100 ml PE and 100 ml distilled water and was shaken vigorously for 2 min. The two layers were allowed to separate. The aqueous layer was discarded and the organic phase was evaporated and dissolved in a known volume of PE.

The concentration of vitamin A was determined by using a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer at 325 nm.

The C_{18} adsorbent in the column was cleaned by tetrahydrofuran (THF) and then rinsed with acetonitrile (ACCN). The cleaned C_{18} adsorbent was dried with nitrogen

gas and left on the column with a cover on the top. The dried C₁₈ adsorbent can be used again.

AOAC Procedure

The AOAC procedure using alumina column chromatography for determination of vitamin A in margarine was applied to milk and infant formula.

The combined ethreal solution from the extraction procedure was evaporated completely in a rotary evaporator. The residue was dissolved in PE.

Standardized alumina and standardized alkaline alumina were prepared according to the AOAC manual (AOAC, 1980). A 10 x 300 mm (I.D. x L) chromatographic column was packed with adsorbant by gravity and slight tapping. Standard alumina was added to height of 1 cm; a 2 cm segment of alkaline alumina was added; 4 cm of standard alumina were added, and finally 1 cm of sodium sulfate was added at the top. After the column was properly packed, the column was wetted with PE.

The sample solution was loaded on the column. The sample flask was rinsed with another portion of PE and applied to the column. As the last of the solution disappeared into the column, portions of 16% ethyl ether in PE were added. The elution was continued with portions of 25% ethyl ether in PE. The column was regularly examined with a U.V. lamp, and the progress of the

fluorescent retinol band was observed. All of the retinol elute was collected and evaporated to dryness in a rotary evaporator. The retinol residue was dissolved in PE, and the absorbance was measured at 325 nm with Perkin-Elmer Lambda 4B UV/VIS Spectrophotometer

HPLC Procedure

The HPLC instrumentation consisted of a Waters model 6000A solvent delivery system (Waters Assoc, Milford, MA), a Waters U6K injector system, a Perkin-Elmer LC-85 spectrophotometric detector LC Auto-Control set at 325 nm and a Perkin-Elmer LC1-100 laboratory computing integrator. A 25 cm x 4.0 mm column containing 10 μ m reverse phase C₁₈ (E. Merk, West Germany) was used in this study.

The chromatographic mobile phase consisted of a mixture of HPLC grade acetonitrile (ACCN), dichloromethane (DCM), and methanol (MEOH) (Fisher Scientific, Medford, MA). All solvents were filtered through a <0.45 μ m membrane filter (Gelman, Ann Arbor, MI). Acetone and PE were distilled before use.

Standards and Standard Curves

Crystalline retinol was purchased from Sigma Chemical Company (St. Louis, MO). Retinol was purified several times according to the procedure described in Arroyava et

al. (1982). The concentration of the purified retinol was measured by a spectrophotometer set at 325 nm. The extinction coefficient for all trans retinol in petroleum ether is 1830 (Barua et al., 1973). The purified retinol was dissolved in chromatographic solvent system (ACCN:DCM:MEOH 70:20:10) (Nelis and DeLeenheer 1983). Peak areas were measured by spectrophotometer set at 325 nm for retinol.

Recovery of Retinol

The recovery was performed by adding a known quantity of vitamin A in the form of retinyl palmitate to the sample. The percentage recovery was calculated by dividing the obtained value of retinol from the spiked one by the original value of retinol for the same sample.

Statistics

The results are expressed as mean \pm SD and compared using the F-test. Correlation coefficients and coefficient of variation between the AOAC, HPLC and reverse phase open column estimate in retinol were calculated.

RESULTS and DISCUSSION

The retinol content of milk and infant formula as determined by the three methods, are given in Table 1. Each sample was analyzed in six replicates and the mean and standard deviation were determined. These data indicate that similar values were obtained using the three methods. The differences among these methods were not statistically significant at ($P > 0.05$), when compared over a different set of samples. Retinol correlation coefficient between the RP-C₁₈ and HPLC estimates and RP-C₁₈ with AOAC estimates were 0.988 and 0.999 respectively at 0.01 probability. The retinol content of milk and infant formula as determined by the three methods were found to be similar to those values reported by other investigator as shown in Table 2.

Figure 2 presents a typical chromatogram for retinol. Retention time of retinol was 2.45 minutes. Peak identification was based on retention time and comparison with the standard as well as Co-chromatography with the standard. For quantification, the peak areas were measured. A linear relationship was observed between concentration and peak areas.

A comparison of reverse phase open column system with both HPLC and AOAC methods is given in Table 3. The average values of RP-C₁₈ were compared by dividing each value with both average value obtained by HPLC and AOAC methods. The comparison between RP-C₁₈ and HPLC method showed values ranging from 0.984 to 1.010 with an average value of 0.995, indicating that the two methods are equal since the value is close to one. The standard deviation of 0.009 and a coefficient of variation of 0.90% were obtained with less than 1.5% variation in results found between these two methods indicating that they are comparable with each other.

The comparison between RP-C₁₈ and AOAC method also presented in Table 3. The actual values ranged from 0.984 to 1.034 with an average value of 1.008 indicating that the two methods are equal since the value is close to one. The standard deviation of 0.017 and a coefficient of variation of 1.7% were obtained with less than 2.6% variation in results found between these two methods, indicating that they are comparable with each other.

The recovery for vitamin A from milk and infant formula was determined by the three methods and found to be 98%, 99% and 97% for RP-C₁₈ HPLC and AOAC respectively.

Conclusion

The RP-C₁₈ open column has several advantages over other methods for the determination of retinol in milk and infant formula such as:

1 - Time: Sample can be analyzed within 10-15 min, which is comparable to HPLC and much shorter than that of the AOAC method (35 min).

2 - Stability: The degradation of vitamin A can be avoided under nitrogen condition and a nonpolar adsorbents, where as in the AOAC method cis-trans isomers can be formed due to long exposure to solvents, polar adsorbent (alumina), light and oxygen.

3 - Ease: The RP-C₁₈ is very simple to operate and does not need any special techniques or equipment other than a spectrophotometer.

4 - Comparability: The study showed that the RP-C₁₈ method is comparable with both AOAC and HPLC methods.

5 - Cost: Solvents required for RP-C₁₈ method are less expensive than that required for HPLC when a mixture of ACCN/DCM/MEOH is used as a mobile phase for HPLC and can be comparable when a mixture of MEOH and water is used as a mobile phase (Table 4). The RP-C₁₈ adsorbent cost less than \$10 and is reusable, the AOAC adsorbent cost \$1.30 and is not reusable, while the HPLC column cost several hundred dollars, but can be used for hundreds of analysis.

6 - Accurate: The RP-C₁₈ method gave a good recovery for vitamin A in milk and infant formula with a value close to HPLC and closer to that of the AOAC method.

µg/100 gr

Sample	Standard Phase	HPLC	AOAC
Whole Milk	25.80 ± 2.24 ^a	24.76 ± 1.78	25.78 ± 1.41
Skim Milk	47.20 ± 1.87	47.46 ± 1.78	47.38 ± 1.76
2% Fat Milk	28.30 ± 1.82	28.38 ± 2.87	28.17 ± 2.78
1% Fat Milk	61.18 ± 2.64	61.71 ± 2.18	62.48 ± 1.88
Infant 1	87.91 ± 7.21	81.87 ± 8.98	82.13 ± 8.98
Infant 2	102.27 ± 1.78	99.25 ± 2.81	97.91 ± 1.81
Infant 3	84.31 ± 1.78	81.55 ± 2.18	88.27 ± 8.11
Formula	87.91 ± 2.14	87.27 ± 2.14	88.21 ± 2.78

^a Mean ± S.E.

Table 1. Retinol Content of milk and Infant Formula Determined by Reverse Phase Open Column System, HPLC and AOAC Methods.

Sample	ug/100 gm		
	Reverse Phase	HPLC	AOAC
Whole Milk	25.89 ± 3.10*	26.26 ± 3.25	25.70 ± 3.43
Skim Milk	47.28 ± 1.39	47.65 ± 1.79	47.10 ± 1.80
2% Fat Milk	68.22 ± 1.83	69.35 ± 2.67	67.12 ± 1.94
1% Fat Milk	61.10 ± 0.64	61.75 ± 2.16	60.48 ± 1.46
Enfamil	82.41 ± 7.21	81.97 ± 6.83	83.13 ± 4.96
SMA	100.37 ± 1.79	99.35 ± 2.43	97.91 ± .91
Isomil	88.91 ± .30	89.65 ± 2.02	90.32 ± 4.15
Prosobee	87.43 ± 2.54	87.37 ± 0.29	84.55 ± 0.78

* Mean ± S.d.

Table 2. Retinol Content of milk and Infant Formula Reported by Other Investigators.

Sample	ug/100 gm	Reference
Whole Milk	18-31.00	Koh (1984)
Skim Milk	44.00	Thompson and Maxwell (1977)
Skim Milk	61.62 - 63.34	Mills (1985)
2% Fat Milk	68.63-69.73	Mills (1985)
1% Fat Milk	69.40-70.38	Mills (1985)
1% Fat Milk	19-47	Koh (1984)
1% Fat Milk	61.54	Leveille et al. (1983)
Infant Formula	60.63-107.21	Landen (1982)
Infant Formula	66.00-108.20	Thompson and Maxwell (1977)

Table 3. Comparison of Reverse Phase Open Column System with HPLC and AOAC Analysis

Sample	RP-C ₁₈ /HPLC	RP-C ₁₈ /AOAC
Whole Milk	0.986	1.007
Skim Milk	0.992	1.004
2% Fat Milk	0.984	1.016
1% Fat Milk	0.988	1.001
Enfamil	1.005	0.991
SMA	1.010	1.025
Isomil	0.992	0.984
Prosobee	1.001	1.034
	$\bar{x} = 0.995$	$\bar{x} = 1.008$
	SD = 0.009	SD = 0.017
	CV = 0.90%	CV = 1.7%
	r = 0.988	r = 0.999

Table 4. The Solvents Cost and time required for the determination of vitamin A by the three methods.

	RP-C ₁₈	HPLC	AOAC
Solvent cost*	\$1.78	\$3.78	\$1.22
Time	10-15 min	10-15 min	35 min

* The mobile phase consisted of MEOH/water for RP-C₁₈, ACCN/DCM/MEOH for HPLC and ethyl ether/PE for AOAC method.

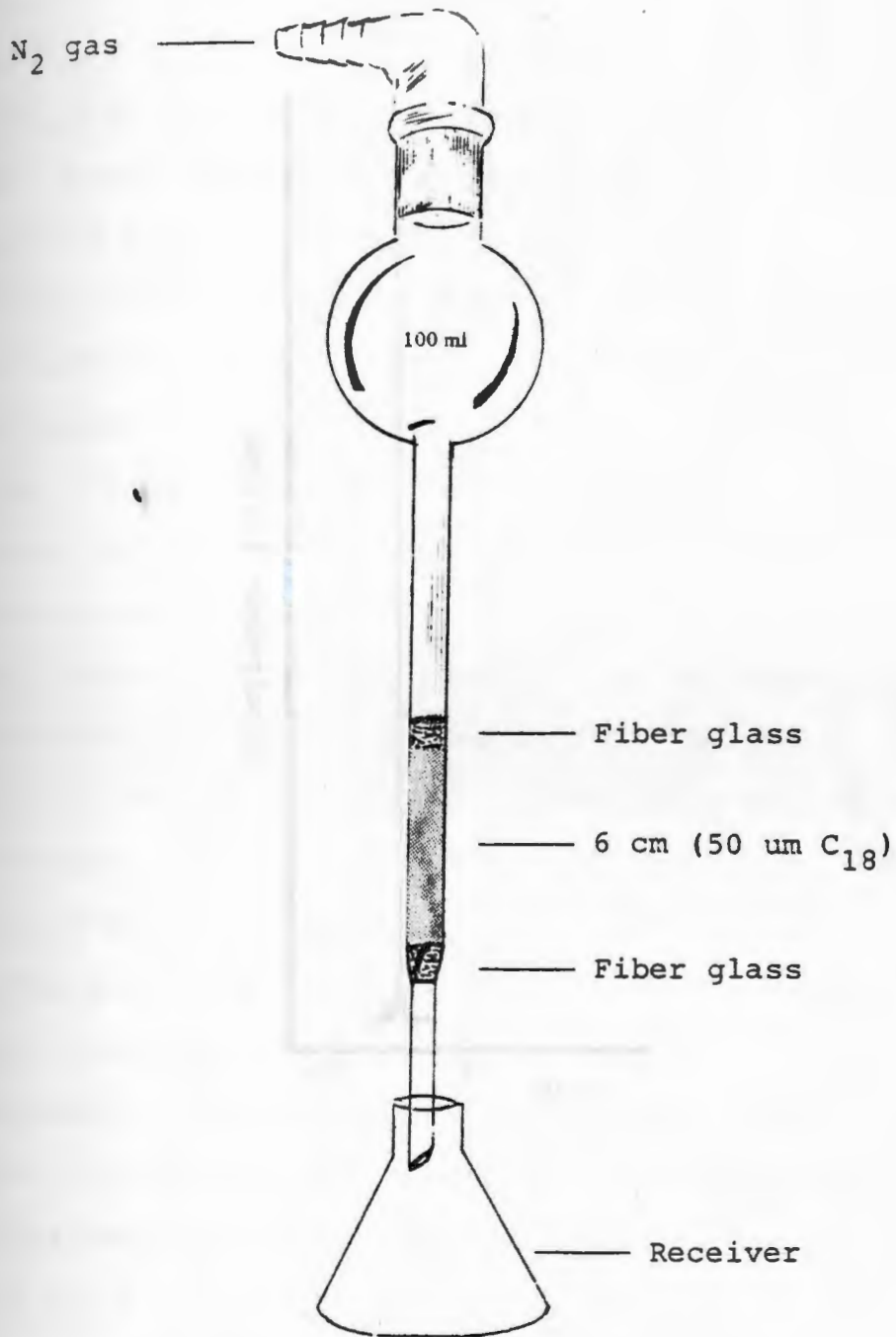


Figure 1. The apparatus of RP - C₁₈ method

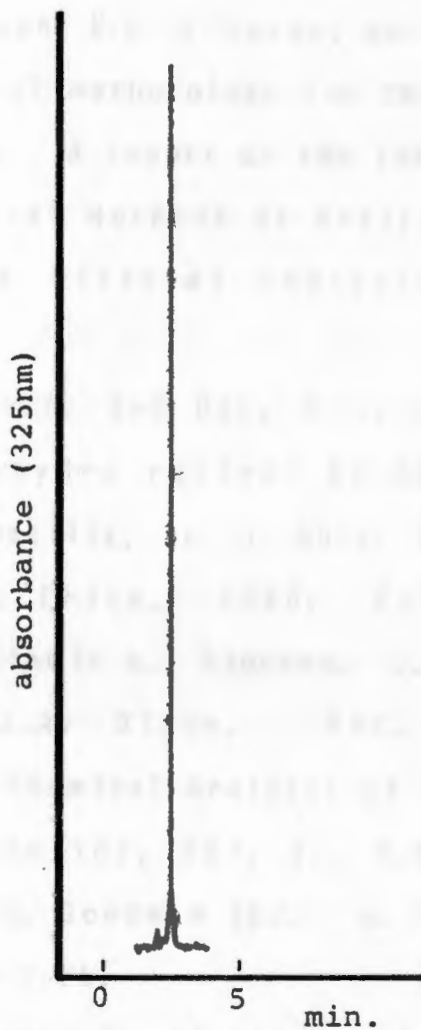


Figure 2. HPLC Chromatogram of Retinol Condition:
 Acetonitrile:Dichloromethane:Methanol
 70:20:10; Flow rate, 1.5 ml/min.

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MANUSCRIPT II

**REVERSE PHASE OPEN COLUMN SYSTEM FOR THE
DETERMINATION OF RETINOL IN MARGARINE,
EGG YOLK AND LIVER**

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ABSTRACT

A simple, inexpensive and comparable method has been developed for the determination of the retinol content of egg yolk, margarine and liver of lamb and chicken. The sample was saponified and extracted with ethyl ether. Separation was achieved on a reverse phase open column, with a mobile phase consisting of methanol and water. The method was statistically evaluated and compared with both the AOAC and HPLC methods. The recovery were 96%, 97% and 96% for RP-C₁₈, HPLC and AOAC respectively. Retinol values obtained by this method were very similar to that of AOAC and HPLC methods. The mean triplicates of each value obtained by reverse phase open column was divided by the mean values obtained by both AOAC and HPLC for the same sample. An average value of 0.997 with a coefficient of variation of 0.31 % between the RP-C₁₈ and the HPLC methods was obtained, also an average value of 0.997 with a coefficient of variation of .70% was obtained between the RP-C₁₈ and AOAC methods. The mean value obtained by this method did not differ significantly with the other methods. Retinol correlation coefficient between RP-C₁₈ and HPLC estimates and RP-C₁₈ with AOAC estimates was 0.999. The RP-C₁₈ method is rapid, simple and may be a useful method for the determination of retinol content in food products.

INTRODUCTION

In the United States, as in many other countries, margarine is fortified with vitamin A in the form of retinyl palmitate which is often the main source of vitamin A activity added to margarine, but sometimes the pigment **B**-carotene accounts for a substantial fraction or all of the vitamin A activity of margarine (Thompson et al. 1980).

A considerable effort was expended for many years on the development of a chromatographic technique for the analysis of vitamin A in margarine which culminated in the publication of an AOAC method specifying an alumina column and an alternative Canadian procedure specifying a polyethylene glycolimpregnated column. Both methods have been reported to be a time-consuming and they require skill and experience (Thompson and Maxwell 1977). These chromatographic methods may or may not result in complete resolution of closely related compounds, and in some cases cis-trans isomers are formed due to the long exposure to solvents, adsorbents, light and oxygen (Targan et al., 1969).

In 1977 Thompson and Maxwell introduced a reverse phase HPLC system for the determination of vitamin A from saponified margarine.

Thompson et al (1980) described a method for the

determination of vitamin A in margarine that eliminates the saponification step, including washings and evaporation of extracts and determined vitamin A directly as retinyl palmitate in hexane extracts of margarine by using high performance liquid chromatography (HPLC). The disadvantages of HPLC are that it is expensive to purchase and maintain, and is not available to nutritional scientists in many parts of the world.

Vitamin A is found in animal products such as liver of lamb and chicken as mixed esters of vitamin A mainly as retinyl palmitate and in egg yolk as retinol with lesser amounts of retinyl esters and retinaldehyde (Parrish, 1977). The colorimetric method of the Carr-Price test (1926) in which vitamin A reacts with antimony trichloride in chloroform yielding a blue color has been described for the determination of vitamin A in egg yolk and chicken liver (Al-Hasani and Parrish 1972). The trifluoroacetic acid reagent has been used for the determination of vitamin A in liver sample from rats by a colorimetric procedure (Sweeney and Marsh 1974). The Carr-Price test (1926) was also employed for the determination of vitamin A in lamb liver by Bhushn et al. (1981). The colorimetric method has disadvantages which include a rapidly fading blue color, sensitivity of the reagent to moisture, interference from carotenoids, and cost of the reagent (Parrish 1977).

A1-Abdulaly and Simpson (1986) established that packing the HPLC adsorbent (50 μm C₁₈) in an open column and eluting the sample with an aqueous, isocratic solvent system (methanol/water 90/10) as the mobile phase, gave good separation of retinol from the extracted sample and the value of retinol by this method was similar to that of the HPLC and AOAC methods for both milk and infant formula.

There is clearly a need for a method that has the simplicity of the AOAC method coupled with the accuracy and speed of the HPLC methods for the determination of retinol content in margarine, egg yolk and liver of chicken and lamb.

In this study, the concept of aqueous reverse-phase chromatography on highly retentive packing materials was applied in the open column, packed with nonpolar small particles (C₁₈) as adsorbents for the analysis for retinol in margarine, egg yolk and liver of chicken and lamb. An aqueous, isocratic solvent system was used as the mobile phase which made the chromatography simple. Nitrogen gas was used to pressurize the column (10 psi) and a conventional spectrophotometer was used for quantification. A comparison of the analysis and recovery for vitamin A by reverse phase open column system, HPLC and AOAC was done for margarine, egg yolk and liver of chicken and lamb.

MATERIALS AND METHODS

Sampling:

Margarine, eggs and liver of chicken were obtained from local supermarkets. A lamb liver was obtained from a slaughterhouse in Rhode Island.

Preparation of Sample:

I. Margarine:

Five grams of margarine was dissolved in hexane in 50 ml volumetric flask. The solution was diluted to volume, shaken and then kept in the dark. A clear solution was obtained after the insoluble materials were settled. A 5 ml aliquot was evaporated to dryness in 100 ml round bottom flask. Water (5 ml) was added to flask containing margarine. Methanol (10 ml) and 5 ml 80% KOH solution were added to the flask. The alkaline mixture was shaken and immersed in 70°C water bath for 30 min. with occasional mixing (Thompson and Maxwell, 1977).

II. Egg Yolk and liver

One gram of sample was ground with anhydrous sodium sulfate to a dry powder in a mortar. The lipids were extracted from the dried material with ethyl ether until no fluorescence appeared to the extract. The extract was

filtered to remove insoluble material, and then evaporated to dryness in a round bottom flask using a rotary evaporator (Hinds et al. 1968). Sufficient methanol was added to the residue to dissolve it and then 60% (W/V) aqueous KOH was added, 1 ml to every 10 ml methanolic solution. The alkaline mixture was shaken and immersed in a 70°C water bath for 30 min. with occasional mixing (Bauernfeind, 1972).

Extraction:

The saponified sample was cooled and transferred to 500 ml separatory funnel. Ethyl ether (50 ml) was added and shaken vigorously. Water was added slowly after 5 min. PE (10 ml) was added and layers were allowed to separate. The aqueous layer was drawn off and extracted three times with ethyl ether. The ethyl ether solutions were then combined in a separatory funnel and washed free from alkali by repeated additions of water. The resultant aqueous layer were discarded. The ethyl ether phase was filtered through Na_2SO_4 to remove moisture, and evaporated to complete dryness in a rotary evaporator. The residue was dissolved in small amount of PE, transferred to small vials and stored under nitrogen gas at -20°C until analysis (Mills 1985).

Chromatography:

Reverse-phase open column chromatographic method

Apparatus. A glass column (1.5 cm x 20 cm) fitted with a sintered disc at the bottom and a 100 ml reservoir, (Kontes Glass Co. #K-420000), was filled with 6 cm of the 50 μm C_{18} adsorbent particles (Separation Technology, Wakefield, RI). The top surface of the adsorbent was covered with a piece of glass wool. Nitrogen gas from a cylinder was used to pressurize the column (10 psi) (Fig. 1). The column was prewetted with the eluting solvent which consisted of a mixture of distilled water and methanol (10:90). The extracted sample was evaporated then dissolved in the eluting solvent, and transferred to the column. Vitamin A was eluted with a 100 ml of the mobile phase. The solution was transferred to a separatory funnel containing 100 ml PE and 100 ml distilled water and was shaken vigorously for 2 min. The two layers were allowed to separate. The aqueous layer was discarded and the organic phase was evaporate and dissolved in a known volume of PE.

The concentration of vitamin A was determined by using a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer at 325 nm.

The C_{18} adsorbent in the column was cleaned after the run by tetrahydrofuran (THF) and then rinsed with acetonitrile (ACCN). The cleaned C_{18} adsorbent was dried

with N₂ gas and left on the column with a cover on the top and used for the next run.

AOAC Procedure

The AOAC procedure using alumina column chromatography for determination of vitamin A in margarine was applied to margarine, egg yold and liver.

The combined ethreal solution from the extraction procedure was evaporated completely in a rotary evaporator. The residue was dissolved in PE.

Standardized alumina and standardized alkaline alumina were prepared according to the AOAC (AOAC, 1980). A 10 x 300 mm (I.D. x L) chromatographic column was packed with adsorbant by gravity and slight tapping. Standard alumina was added to height of 1 cm; a 2 cm segment of alkaline alumina was added; 4 cm of standard alumina were added, and finally 1 cm of sodium sulfate was added at the top. After the column was properly packed, the column was wetted with PE.

The sample solution was loaded on the column. The sample flask was rinsed with another portion of PE and applied to the column. As the last of the solution disappeared into the column, portions of 16% ethyl ether in PE were added. The elution was continued with portions of 25% ethyl ether in PE. The column was regularly examined with a U.V. lamp, and the progress of the

fluorescent retinol band was observed. All of the retinol elute was collected and evaporated to dryness in a rotary evaporator. The retinol residue was dissolved in PE, and the absorbance was measured at 325 nm in the Spectrophotometer

HPLC Procedure

The HPLC instrumentation consists of a Waters model 6000A solvent delivery system (Waters Assoc, Milford, MA), a Waters U6K injector system, and a Perkin-Elmer LC-85 spectrophotometric detector LC Auto-Control set at 325 nm and connected to Perkin-Elmer LC1-100 laboratory computing integrator. A 25 cm x 4.0 mm column containing 10 μ m reverse phase C₁₈ (E. Merk, West Germany) was used in this study.

The chromatographic mobile phase consisted of mixture of HPLC grade acetonitrile (ACCN), dichloromethane (DCM), and methanol (MEOH) (Fisher Scientific, Medford, MA). All solvents were filtered through a <0.45 μ m membrane filter (Gelman, Ann Arbor, MI). Acetone and PE were distilled before use.

Standards and Standard Curves

Crystalline retinol was purchased from Sigma Chemical Company (St. Louis, MO). Retinol was purified several times according to the procedure described in Arroyava et

al. (1982). The concentration of the purified retinol was measured by a spectrophotometer set at 325 nm. The extinction coefficient for all trans retinol in petroleum ether is 1830 (Barua et al., 1973). The purified retinol was dissolved in chromatographic solvent system (ACCN:DCM:MEOH 70:20:10) (Nelis and DeLeenheer, 1983). Peak areas were measured by spectrophotometer set at 325 nm for retinol. A standard curve was prepared from peak area versus a known concentration of injected retinol.

Recovery of Retinol

The recovery for retinol was performed by adding a known quantity of vitamin A in the form of retinyl palmitate to the sample. The percentage recovery was calculated by dividing the obtained value of retinol from the spiked one with the original value of retinol for the same sample.

Statistics

The results are expressed as mean \pm SD and compared using the F-test. Correlation coefficients and coefficient of variation between the AOAC, HPLC and reverse phase open column estimate in retinol were calculated.

RESULTS AND DISCUSSION

Retinol content of margarine, egg yolk and liver of lamb and chicken as determined by the three methods are given in Table 1. Each sample was analyzed in triplicate and an average and standard deviation were determined. These data indicate that similar values were obtained using the three methods. The differences among these methods were not statistically significant at ($P > 0.05$), when compared over a different set of samples. Margarine was labelled as containing 10 percent of the U.S. RDA in 14 grams which is equivalent to 1071 ug per 100 gm margarine (Krause and Mahan 1979). The study showed that vitamin A content ranged from 523. to a high of 908.0 ug per 100 gm for the three different brands of margarine by the three methods. The vitamin A activity of margarine is due not only to added retinyl palmitate but to the presence of **B**-carotene (vitamin A precursor), thus the actual value would be higher.

The retinol content of analyzed samples was within a range values reported by other investigators as shown in table 2. Fig. 2 presents a typical chromatogram for retinol with a retention time of 2.42 minutes. Peak identification was based on retention time and comparison with the standard as well as co-chromatography with the standard. For quantification, the peak areas were

measured. A linear relationship was observed between concentration and peak areas.

A comparison of reverse phase open column system (RP-C₁₈) with both HPLC and AOAC methods is given in table 3. The average values of RP-C₁₈ were compared by dividing each value with both average value obtained by HPLC and AOAC methods.

The comparison between RP-C₁₈ and HPLC method showed an actual values ranges from 0.991 to 1.001 with an average value of 0.997, indicating that the two methods are equal since the value is close to one. The standard deviation of 0.0031 and a coefficient of variation of 0.31% were obtained with less than 0.4% variation in results found between these two methods indicating that they are comparable with each other. Retinol correlation coefficient between the RP-C₁₈ and HPLC estimates was 0.998 at 0.01 probability. This indicates that a highly significant correlation between these two methods for the determination of vitamin A from different samples.

The comparison between RP-C₁₈ and AOAC method also presented in table 3. The actual values ranged from 0.993 to 1.010 with an average value of 0.997 indicating that the two methods are equal since the value is close to one. The standard deviation of 0.007 and a coefficient of variation of 0.70% were obtained with less than 1.3% variation in results found between these two methods

indicating that they are comparable with each other. Retinol correlation coefficient between the two methods estimates was 0.999 at 0.01 probability. This indicates a highly significant correlation between these two methods for the determination of vitamin A from margarine, egg yolk and liver of lamb and chicken.

The recovery for vitamin A from the analyzed samples was determined by the three methods and found to be 96.00%, 97% and 96% for RP-C₁₈, HPLC and AOAC respectively. The study showed a very similar value for different methods of vitamin A determination from margarine, egg yolk and liver.

CONCLUSION

The objective of this research was to develop a simple, inexpensive and comparable method, and applying the method for retinol determination in margarine, egg yolk and liver of lamb and chicken.

The sample can be analyzed for retinol content within 15 min., which is comparable to HPLC and much shorter than that of AOAC method. The retinol degradation can be avoided under nitrogen gas conditions and nonpolar adsorbents. In the AOAC method cis-trans isomers can be formed due to long exposure to solvents, polar adsorbent, light and oxygen. The method is very simple to operate and does not need special equipment. In this regard it is similar to the AOAC method and much easier than that of HPLC method. The cost of solvents required for RP-C₁₈ method are less expensive than that required for HPLC when a mixture of ACCN/DCM/MEOH is used as a mobile phase for HPLC and can be comparable when a mixture of MEOH and water is used as a mobile phase (Table 4). The RP-C₁₈ adsorbent cost less than \$10 and is re usable, the AOAC adsorbent cost \$1.30 and is not reusable, while the HPLC column cost several hundred dollars but is reusable.

This study indicates that the RP-C₁₈ method is a good method to analyze margarine, egg yolk and liver of lamb and chicken for retinol content due to its advantages of time saving, comparability and low cost. The values obtained were similar to those obtained by the HPLC and AOAC methods.

Table 1. Retinol content of egg yolk, margarine, and liver as determined by reverse phase open column system, HPLC and AOAC methods.

		ug/100 gm		
Sample		RP-C ₁₈	HPLC	AOAC
Egg Yolk	I.	300.11 ± 2.66	302.91 ± 1.60	301.57 ± 4.05
	II.	323.10 ± 1.25	323.86 ± 1.04	323.44 ± 1.73
	III.	224.05 ± 0.8	224.44 ± 0.58	225.70 ± 1.57
Margarine				
Brand	I.	760.00 ± 7.70	768.54 ± 8.48	769 ± 8.80
Brand	II.	523.71 ± 3.27	524.10 ± 2.88	526.00 ± 3.00
Brand	III.	906.02 ± 4.89	907.17 ± 2.59	908.22 ± 4.04
Liver				
Lamb		44300 ± 0.89	44370 ± 0.93	43870 ± 1.33
Chicken		3520.0 ± 0.12	3540.0 ± 0.14	3560.0 ± 0.08

Table 2. Retinol content of margarine, egg yolk and liver reported by other investigators

Sample	ug/100 gm	Reference	
Margarine	80-1495	Thompson and Maxwell 1977	
	27-982	Thompson et al. 1980	
	521-827	Sivell et al. 1984	
	671 - 1090	Egberg et al. 1977	
Egg yolk	400	Paul and Southgate 1978	
	552.90	Pennington and Church 1985	
	238.57	Leveille et al. 1983	
Liver	Lamb	18100 (3000 - 5,000)	Paul and Southgate 1978
		15,165	Pennington and Church 1985
	Chicken	3633.63	USDA 1975
		4917.42	Pennington and Church 1985
		1213.21	Neamtu, G. et al. 1977.

Table 3. Comparison of reverse phase open column system with HPLC and AOAC analysis

Sample	RP-C ₁₈ /HPLC	RP-C ₁₈ /AOAC
Egg yolk		
I	0.991	0.995
II	0.998	0.999
III	0.998	0.993
Margarine		
Brand I	1.001	0.998
Brand II	0.999	0.996
Brand III	0.998	0.997
Liver		
Lamb	0.998	1.010
Chicken	0.994	0.989
	\bar{x} 0.997	0.997
	SD 0.0031	0.007
	c.v. 0.31%	0.70%
	r 0.998	0.999

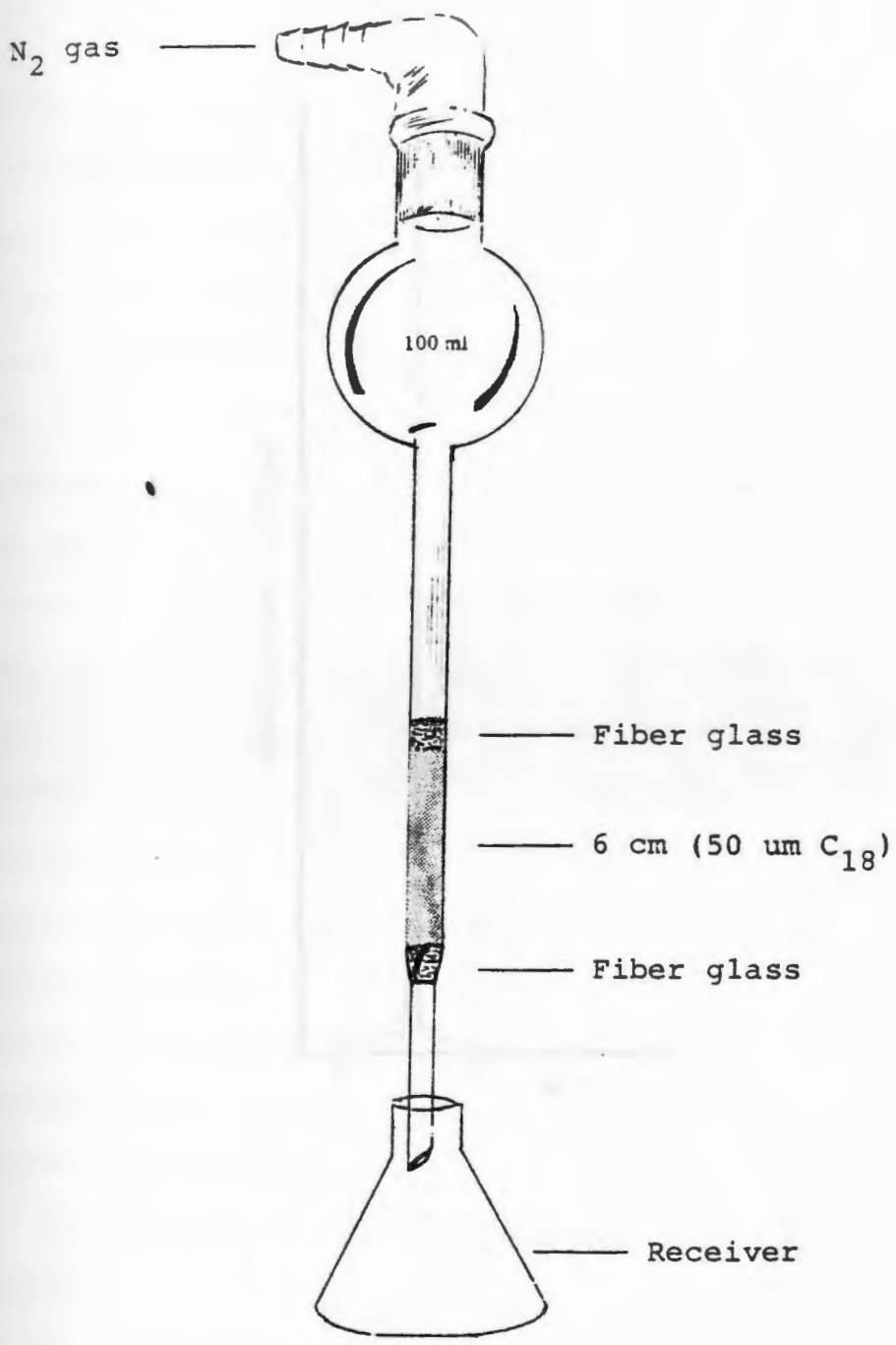


Figure 1. The apparatus of RP - C₁₈ method

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MANUSCRIPT III

ABSTRACT

THE DETERMINATION OF SERUM RETINOL BY REVERSE
PHASE OPEN COLUMN CHROMATOGRAPHY

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ABSTRACT

An isocratic, aqueous reverse phase open column chromatography has been developed to allow simple, rapid and inexpensive determination of retinol concentration in serum. The sample was extracted with hexane. Separation was achieved on a reverse phase open column, with a mobile phase consisting of methanol and water. The method was statistically evaluated and compared with HPLC method. Retinol values obtained by this method were similar to that of HPLC. An average value of 0.991 with a coefficient of variation of 4.94% between the RP-C₁₈ open column and the HPLC methods was obtained. The mean value obtained by this method did not differ significantly with HPLC method. Retinol correlation coefficient between RP-C₁₈ open column and HPLC estimates was 0.963. The RP-C₁₈ open column is rapid, simple, inexpensive and may be a useful method for the determination of retinol content in serum in clinical laboratories that cannot afford to have HPLC instrument.

INTRODUCTION

The quantitative determination of retinol (vitamin A) in human blood is important for the evaluation of vitamin A status of individuals and population. Until the late 1970's, retinol blood concentrations were determined by spectrophotometric methods, colorimetric assays or fluorescence assays (Nierenberg, 1984).

In spectrophotometric method, it is assumed that nothing except vitamin A is absorbed in the 325 to 330 nm region. Various lipids, sterols and vitamin E and D absorb in the same general region of the spectrum as vitamin A (Parrish, 1977). The fluorometric method does not always result in a complete separation of vitamin A from other fluorescent components, time consuming and in some cases cis-trans isomers are formed due to the long term exposure of vitamin A to oxygen, light, absorbents and solvents (Targan et al., 1969). The colorimetric method has disadvantages which include rapidly fading blue color, sensitivity of the reagent to moisture, interference from carotenoids, time consuming and cost of the reagent (Parrish, 1977).

The recent application of high pressure liquid chromatography (HPLC) to analyses of biological material has provided the nutritional biochemist with highly useful

techniques with a wide variety of applications. The advantages of HPLC over other methods include its speed, high resolution of closely related compounds, nondestructive conditions, applicability to very small samples and selective quantitation of retinol in blood based upon retinol's high intrinsic UV absorption (Bieri et al., 1979) (Nierenberg, 1984).

The disadvantages of HPLC are that it is expensive to purchase and maintain, and is not available to nutritional scientists in many parts of the world.

Al-Abdulaly and Simpson (1986) described the use of an HPLC adsorbent (50 μm C_{18}) in an open column and elution the sample with an aqueous, isocratic solvent system (methanol/water 90/10) as the mobile phase. This procedure gave good separation for retinol from the extracted milk and infant formulas as the value was similar to that of the HPLC and AOAC method.

The concept of aqueous reverse-phase chromatography was applied in the open column, packed with 50 μm C_{18} particles as adsorbents for analysis of retinol in blood. An aqueous, isocratic solvent system was used as the mobile phase. Nitrogen gas was used to pressurize the column (10 psi) and a conventional spectrophotometer was used for quantification. The comparison of the analysis for retinol by reverse phase open column system and HPLC was done for blood samples.

Materials and Methods

Sampling

Blood was obtained in heparinized tubes by ventipuncture by nurses at North Kingstown Laboratory, Inc. (North Kingstown, Rhode Island) from volunteer graduate students at U.R.I. Blood samples were immediately placed in an insulated box with ice and transported to the laboratory.

Separation of Serum

The serum was separated by centrifugation of blood at 3000 RPM for 15 minutes in a cold room. Serum was transferred to a test tube, the head space was filled with nitrogen gas and the tube was tightly sealed. The serum was stored at -20°C until analysis.

Extraction of serum

The extraction procedure used was similar to that outlined by Schindler et al. (1985) and Woollard G.A. and Woollard D.C. (1984) with a modification. 2 ml serum sample was placed in a Kimax 150 x 5 mm glass centrifuge tube equipped with a teflon-lined screw cap. 2 ml ethanol was added and the contents were vortexed to ensure protein precipitation. 2 ml water was then added followed by 4.0

ml of hexane. The mixture was vortex mixed for 1 min and subsequently centrifuged for 5 min. at 2000 RPM. The hexane phase was quantitatively removed and the extraction process repeated once. The hexane extracts were combined and evaporated to dryness in a rotary evaporator. The sample was dissolved in a small amount of petroleum ether and stored under nitrogen gas in small vials, at -20°C until analysis.

Chromatography:

Reverse-phase open column chromatographic method

Apparatus. A glass column (1.5 cm x 20 cm) fitted with a sintered disc at the bottom and a 100 ml reservoir, (Kontes Glass Co. #K-420000), was filled with 6 cm of the 50 um C₁₈ adsorbent particles (Separation Technology, Wakefield, RI). The top surface of the adsorbent was covered with a piece of glass wool. Nitrogen gas from a cylinder was used to pressurize the column (10 psi) (Fig. 1). The column was prewetted with the eluting solvent which consisted of a mixture of distilled water and methanol (10:90). The extracted sample was evaporated then dissolved in the eluting solvent, and transferred to the column. Vitamin A was eluted with a 100 ml of the mobile phase. The solution was transferred to a separatory funnel containing 100 ml PE and 100 ml distilled water and was shaken vigorously for 2 min. The two layers were

allowed to separate. The aqueous layer was discarded and the organic phase was evaporate and dissolved in a known volume of PE.

The concentration of vitamin A was determined by using a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer at 325 nm.

The C₁₈ adsorbent in the column was cleaned after the run by tetrahydrofuran (THF) and then rinsed with acetonitrile (ACCN). The cleaned C₁₈ adsorbent was dried with N₂ gas and left on the column with a cover on the top. The dried C₁₈ adsorbent could be used again.

HPLC Procedure

The HPLC instrumentation consisted of a Waters model 6000A solvent delivery system (Waters Assoc, Milford, MA), a Waters U6K injector system, a Perkin-Elmer LC-85 spectrophotometric detector LC Auto-Control set at 325 nm and a Perkin-Elmer LC1-100 laboratory computing integrator. A 25 cm x 4.0 mm column containing 10 um reverse phase C₁₈ (E. Merk, West Germany) was used in this study. The chromatographic mobile phase consisted of mixture of HPLC grade acetonitrile (ACCN), dichloromethane (DCM), and methanol (MEOH) (Fisher Scientific, Medford, MA). All solvents were filtered through a <0.45 um membrane filter (Gelman, Ann Arbor, MI). Acetone and PE were distilled before use.

Standards and Standard Curves

Crystalline retinol was purchased from Sigma Chemical Company (St. Louis, MO). Retinol was purified several times according to the procedure described in Arroyava et al. (1982). The concentration of the purified retinol was measured by a spectrophotometer set at 325 nm. The extinction coefficient for all trans retinol in petroleum ether is 1830 (Barua et al., 1973). The purified retinol was dissolved in chromatographic solvent system (ACCN:DCM:MEOH 70:20:10) (Nelis and DeLeenheer, 1983). Peak areas were measured by spectrophotometer set at 325 nm for retinol. A standard curve was prepared from peak area versus a known concentration of injected retinol.

Statistics

The results are expressed as mean \pm SD and compared using the F-test. Correlation coefficients and coefficient of variation between HPLC and reverse phase open column estimate in retinol were calculated.

Results and Discussion

Blood samples were obtained from healthy volunteer students and assayed for retinol content by reverse phase open column chromatography and compared with HPLC method.

Figure 2 shows the scanning profile for the extracted serum before it was applied to the RP-C₁₈ open column. As can be seen, the extracted serum contains carotenoids and some unidentified contaminants which absorb in the same general region of the spectrum as vitamin A.

The chromatography on an open column containing (50 μ m C₁₈) was carried out for the extracted serum, and a typical scanning profile is shown in Figure 3. As can be observed from Figure 3, separation of retinol from serum can be achieved using this chromatographic system while the carotenoids and other interfering substances were retained completely on the column. The retinol concentration can be determined from the absorbance obtained by the spectrophotometer.

The retinol content of serum as determined by reverse phase open column (50 μ m C₁₈) and HPLC method is given in Table 1. Each blood sample was analyzed in triplicate and the mean and standard deviation were determined. These data indicate that similar values were obtained using the two methods. The differences among these methods were not statistically significant at ($P > 0.05$), when compared

over a different set of samples. Retinol correlation coefficient between the RP-C₁₈ and HPLC estimates was 0.963 at 0.01 probability. The retinol content of serum as determined by the two methods were found to be similar to those values reported by other investigators as shown in Table 2.

Figure 4 presents a typical chromatogram for retinol. Retention time of retinol was 2.45 minutes. Peak identification was based on retention time and comparison with the standard as well as co-chromatography with the standard. For quantification, the peak areas were measured. A linear relationship was observed between concentration and peak areas.

A comparison of reverse phase open column system with HPLC method is given in Table 3. The average values obtained by RP-C₁₈ were compared by dividing each value with average value obtained by HPLC. The comparison between the two methods showed values ranging from 0.930 to 1.049 with an average value of 0.991, indicating that the two methods are equal since the value is close to one. The standard deviation of 0.049 and a coefficient of variation of 4.49% were obtained with less than 5.8% variation in results found between these two methods indicating that they are comparable with each other.

Conclusion

The retinol content of serum was assayed by both reverse phase open column (50 μ m C₁₈) and HPLC. The study showed the two approaches gave a similar value with no significant difference, correlated well ($r = 0.963$) and were comparable with each other. This good agreement between results by the two techniques indicates that interference substances such as phytofluene was retained on the open column and only retinol was detected in the mobile phase.

In this system, the sample can be analyzed within 15 min. which is comparable to HPLC. The method is very simple and only requires a spectrophotometer for quantification. The solvent grades are less critical for RP-C₁₈ than the HPLC. Like the HPLC the RP-C₁₈ is reusable.

This study indicates that the RP-C₁₈ method is a good method to analyze serum for retinol content. This method can be an alternative technique for clinical laboratories that do not have HPLC or wish to use HPLC for calibration purposes. The system can be available to nutritional scientists in many part of the world. By the use of micro cuvetts and small column the blood sample size could be reduced.

Table 1. Retinol content of serum determined by RP-C₁₈ open column system and HPLC methods.

ug/100 ml		
Sample	RP-C ₁₈	HPLC
1	54.47 ± 1.40	58.67 ± 1.53
2	54.00 ± 1.00	51.47 ± 1.05
3	47.00 ± 0.98	47.00 ± 0.63
4	33.87 ± 0.16	34.38 ± 0.54

Table 2. Retinol content of serum reported by other investigators.

Reference	ug/100 ml serum
Thompson et al. (1971)	31.2
Pollack et al. (1973)	24.5
DeRuyter and DeLeenheer (1976)	58.90
Bieri et al. (1979)	53.8
Koh, Y. (1984)	31.00 (15-46)
Miller and Yang (1984)	34.97 (14.6-62.9)
Nierenberg, D.W. (1984)	44.8
Schindler et al. (1985)	56.4

Table 3. Comparison of reverse phase open column system with HPLC method for retinol analysis in serum.

Sample	RP-C ₁₈ /HPLC
1	0.930
2	1.049
3	1.00
4	0.985
	\bar{x} 0.991
	SD 0.049
	C.V. 4.94%
	r 0.963



Figure 1. The apparatus of RP-C₁₈ method.

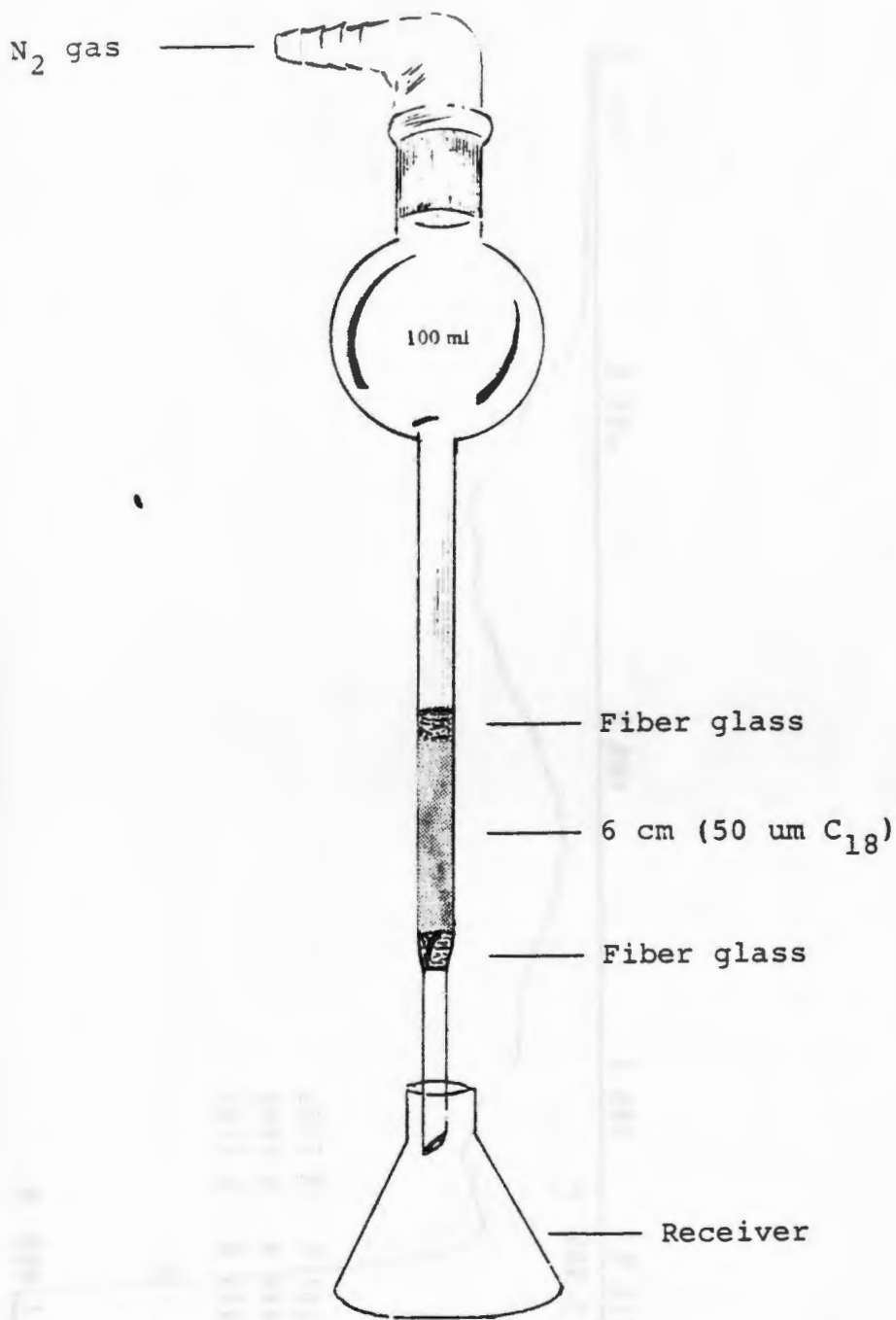


Figure 1. The apparatus of RP - C₁₈ method

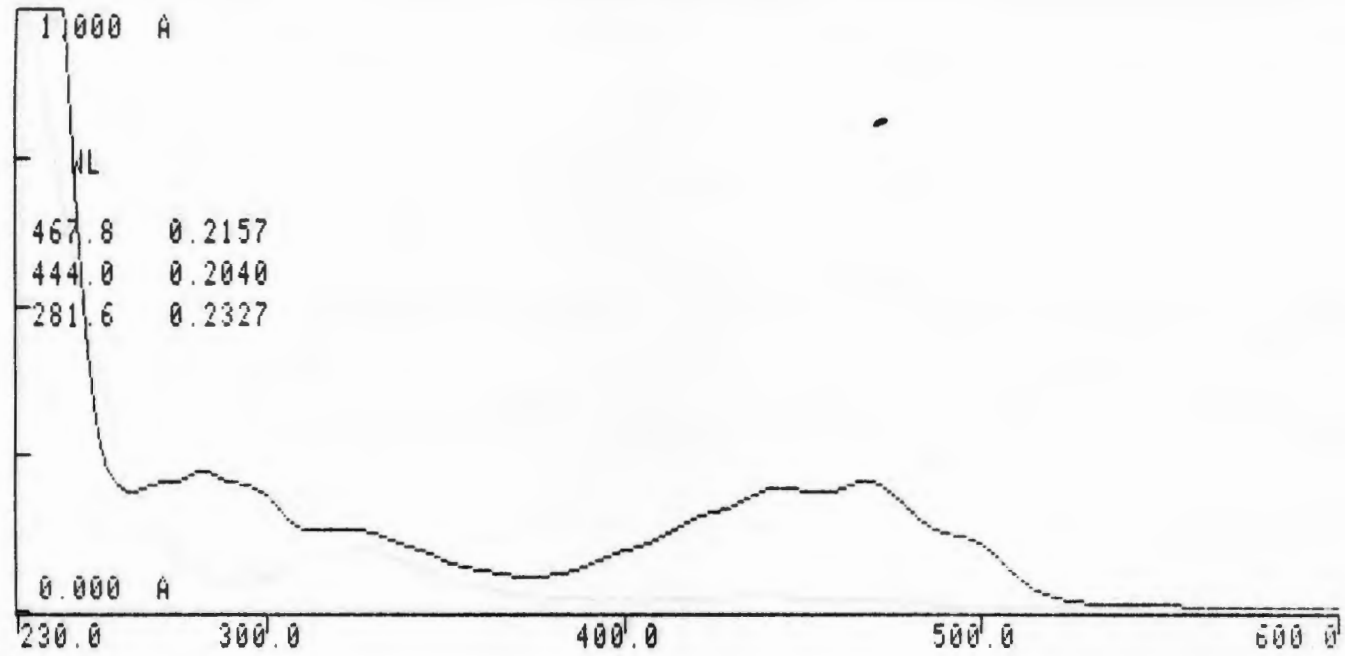


Figure 2. Absorption spectrum of extracted serum in PE

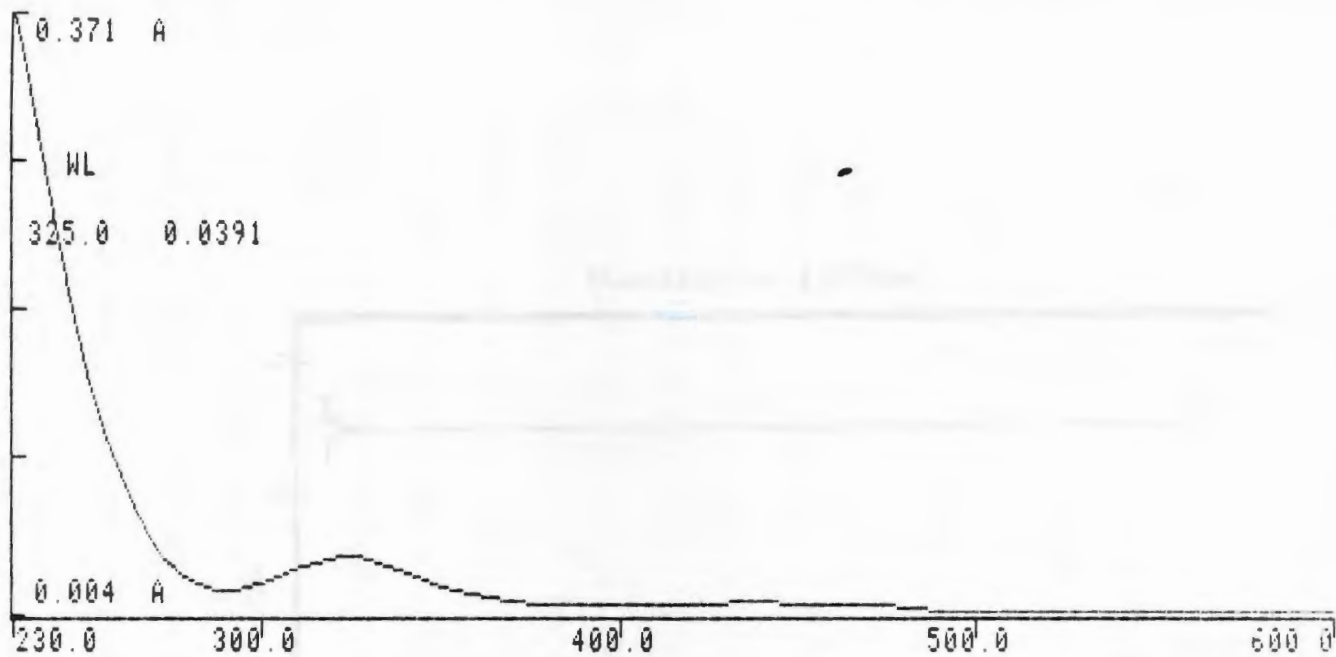


Figure 3. Absorption spectrum of extracted serum after the RP-C₁₈ open column in PE

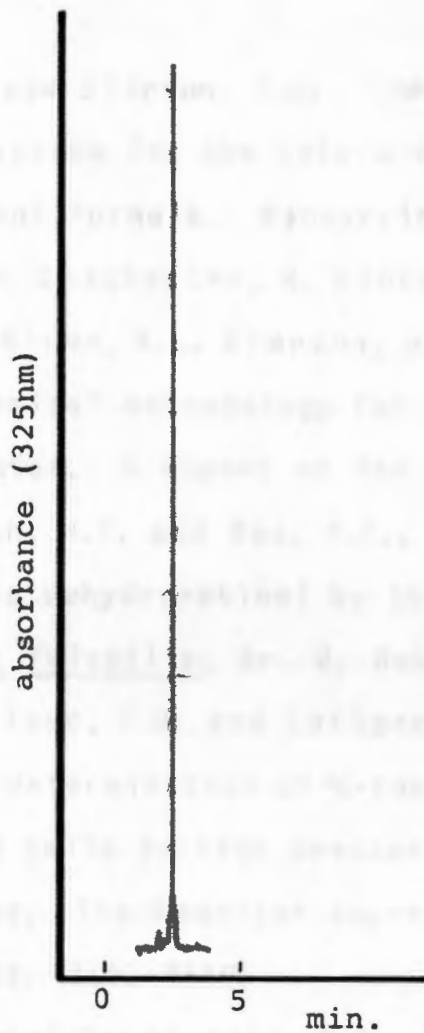


Figure 4. HPLC chromatogram of retinol;
ACCN:MEOH:DCM 70:10:20. Flow rate
1.5 ml/min.

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LITERATURE REVIEW

Vitamin A is an essential nutrient for man since it cannot be synthesized within the body. It is usually supplied in the daily diet either as vitamin A, as carotenoid vitamin A precursors (provitamin A) or as a mixture of the two. Additionally, synthetic preformed vitamin A, identical to that in nature, is available to be taken orally, administered intramuscularly or added to foods consumed by man (Bauernfiend, 1980).

Vitamin A performs several important functions in the body. It is required for vision in dim light and for normal health of epithelial tissues which line the digestive tract, the respiratory tract, the genitourinary tract, the eyes, and the skin. It is needed for normal bone growth and for maintenance of the structure of membranes (Williams and Caliendo, 1984).

Vitamin A is found in animal products such as liver, milk, butter and cheese as mixed esters of vitamin A and in egg yolk as retinol with lesser amounts of retinaldehyde and retinyl esters (Parrish, 1977).

Vitamin A is generally added to milk that is partially or wholly defatted, margarine and infant formulas. It is added in specified amounts to these products in the form of retinyl esters mainly as retinyl palmitate. Retinyl palmitate is often the main source of

vitamin A activity added to these foods, but sometimes the pigment **B**- carotene accounts for substantial fraction or all of the vitamin A activity of margarine (Thompson and Maxwell, 1977).

Vitamin A is found in plasma in two major forms, retinol and its ester. Retinol is about 90% or more under most conditions, the retinyl ester is about 10 to 17 percent (Sommer, et al. 1977).

Since the discovery of the importance of vitamin A, nutritionists have been interested in the vitamin A content of foods and diets, and vitamin A has been determined in many natural and some processed foods containing both vitamin A and provitamin A. Vitamin A determination on foods has assumed greater importance because of the growing interest in nutrient requirements, dietary standards, nutrient labeling and warranties among consumers, processors, and regulatory agencies (Parrish, 1977).

In foods, the concentration of vitamin A is small in comparison to other components that may interfere with analysis or the vitamin A is held so that it cannot be extracted without pretreatment. Saponification, which is an alkaline digestion, is generally used in the vitamin A determination for foods to free the vitamin from the stabilizing matrix, from the lipids in which it might be dissolved, or from substances in the food that might

interfere with the extraction. In this process, the esterified vitamin A is converted to the alcoholic form, retinol, without destroying the vitamin (Parrish, 1977).

Several methods for vitamin A determination in blood and food products have been published, and all these methods depend on the physical and chemical properties of retinol which include the following: it is absorbed at 325 nm, it fluoresces at 480 nm, and it forms colored products with antimony trichloride and trifluoroacetic acid. The most common methods are spectrophotometric, fluorometric, colorimetric, and chromatographic methods (Parrish, 1977).

In the spectrophotometric method vitamin A is determined in the 325 to 330 nm region, either by a direct reading or by the difference in readings before and after vitamin A is destroyed by irradiation. In this method it is assumed that nothing except vitamin A is absorbed in the 325 to 330 nm region. Various lipids, sterols and vitamin E and D absorb in the same general region of the spectrum as vitamin A. Alkaline hydrolysis and chromatographic steps are required to remove these interfering substances. The method of the Association of Official Analytical Chemists (AOAC) for vitamin A in margarine is based on a spectrophotometric reading following alumina column chromatography.

The colorimetric method of the Carr-Price test in

which vitamin A reacts with antimony trichloride in chloroform yielding a blue color is by far the most widely used colorimetric method to determine vitamin A in foods (Carr and Price, 1926). This method has disadvantages which include rapidly fading blue color, sensitivity of the reagent to moisture, interference from carotenoids, and cost of the reagent (Parrish, 1977). A new colorimetric procedure was developed by Neeld and Pearson (1963) based on the blue color produced by trifluoroacetic acid in the presence of vitamin A. The major advantage of the trifluoroacetic acid method is that the blue color is more stable and less susceptible to interference by traces of moisture.

The fluorometric method for vitamin A determination is based on the molecular fluorescence properties of the retinol structure (Thompson et al., 1971, 1978). The presence of fluorescent components other than retinol such as phytofluene and lipids in the extracted food or blood samples raise the problem of nonspecificity. To overcome this problem, Garry et al. (1970) and Pollack et al. (1973) introduced a chromatographic step before fluorometry to remove the interfering substances. Today, the method for determining vitamin A in margarine recognized by the AOAC calls for fluoremetric determination following alumina column chromatography (AOAC, 1980).

The chromatographic methods are not complete analytical methods. They merely separate extracts of sample into components, and spectrophotometric, colorimetric or fluorometric analysis are required to measure the components separated. Both adsorption and partition chromatography are used to remove substances interfering in determination of vitamin A. Other methods include high-pressure liquid chromatography (HPLC) and gas-liquid chromatography (Parrish, 1977).

In column chromatography, a number of adsorbents have been used for separating vitamin A from other fat soluble substances. Neutral alumina was one of the earliest techniques and most commonly used method to separate retinol from its esters (Thompson et al., 1971 and 1978). This method of adsorption chromatography utilizes alumina that has been deactivated, usually with 5 percent water, and most often employed diethyl ether, benzene or acetone in hexane as the eluting solvent. Recoveries vary from 85 to 96 percent for retinol and retinyl esters (Ross and Zilversmit, 1977). Thin-layer and paper (partition) chromatography are generally not adaptable to quantitative determination of vitamin A in foods. They are used for qualitative estimations, checks on homogeneity, forms of vitamin A in extracts and presence of other vitamins and lipids (Parrish, 1977). The most common adsorbents are silica gel and alumina.

The usual conditions under which thin-layer and paper separation are carried out promote oxidations and loss of vitamin A (Sajak et al., 1979). These chromatographic methods may or may not result in complete resolution of closely related compounds, require several hours and in some cases cis-trans isomers are formed due to the long exposure to solvents, adsorbents, light and oxygen. The recoveries are usually in the range of 70 to 85 percent (Targan et al., 1969)

Gas-liquid chromatography has a limited use in the determination of vitamin A because of the instability of retinol to heat. Retinol and retinyl esters were reported to be rapidly dehydrated to anhydroretinol on several gas chromatographic columns even at lower temperatures as low as 150°C. (Frolik and Olson, 1984).

The recent application of high pressure liquid chromatography (HPLC) to the analysis of biological materials has provided the nutritional biochemist with highly useful techniques for a wide variety of applications. The advantages of HPLC over spectrophotometry, fluorometry or gas chromatography include its speed, high resolution of closely related compounds, a recovery for vitamin A in foods of up to 100 percent (Van De Weerdof et al., 1973), a highly sensitive technique capable of detecting nanogram levels of retinol, nondestructive conditions and a simplified methodology

(Frolík and Olson, 1984). The disadvantages of HPLC are that it is expensive to purchase and maintain, is not available to nutritional scientists in many parts of the world, and it requires skilled operators.

Shu-Whei Tsai (1986) reported that packing the HPLC adsorbent (C₁₈) in the open column and eluting it with the isocratic, nonaqueous solvent system (Acetonitrile/methanol/chloroform) as the mobile phase gave good separation of α - and β -carotene from β -cryptoxanthin and lycopene, and the results were the same as that of AOAC method for carrots, and very similar to that of the HPLC method for spinach and peaches.

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