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Validation of an Analytical Method Based on High-Performance Liquid Chromatography for the Determination of Retinol in Chicken Liver

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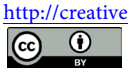


Abstract

Retinol is an unsaturated fat-soluble isoprenoid alcohol. It is essential for the normal functioning of cells, human growth, and development, and it is found in animal sources in the form of palmitate ester. Chicken liver is an excellent source of retinol and is widely used in the diet of people in developing countries to combat Vitamin A deficiency. Several methods for the quantification of retinol in chicken liver have been reported in the literature. Most of them use more than one solvent for the determination and quantification of this micronutrient. A new method was developed based on a reverse phase system and using isocratic elution in a C18 column (5 mm, 250 × 4.6 mm) coupled to a SPD-20A UV-VIS detector at a wavelength of 325 nm with a single mobile phase, methanol. The method is simple and inexpensive, allowing the rapid extraction, determination, and quantification of retinol in chicken liver samples, with an average retention time of 5.2 min at 23˚C, yielding good linearity results (R^2 = 0. 9999), standard stock and freeze–thaw process stabilities of 93.2% and 97.5%, respectively, precision with coefficients of variation below 15%, and recovery coefficients ranging between 93% and 101.2%.

Subject Areas

Analytical Chemistry

Keywords

HPLC, Validation, Retinol, Method, Methanol

1. Introduction

Vitamin A is a fat-soluble micronutrient that comprises a series of compounds

displaying the biological activity of retinol (Figure 1), which exists either as preformed vitamin A (retinol, retinal, retinoic acid, and retinyl esters) or as pro-vitamin A (alpha-carotene, beta-carotene, and beta-cryptoxanthin) (YUYAMA *et al.*, 2012) [1].

Retinol's function is associated with visual, reproductive, and immunological processes. It is also essential for cell differentiation and fetal development. Vitamin A deficiency (VAD) is still considered a global disease, particularly in emerging countries where its diagnosis is closely related to a low socioeconomic status. VAD affects the vision of the patient, initially with night blindness, and leading to possible xerophthalmia and keratomalacia symptoms. When untreated, these symptoms become irreversible, resulting in vision loss (BRASIL, 2009 [2]; GALLAGHER, 2010 [3]; SAUNDERS *et al.*, 2009 [4]; YUYAMA *et al.*, 2012 [1]).

Brazil presents a large number of cases of VAD in the Northeast region and in some regions of the Southeast, where the populations' diet is poor in both quality and quantity as a result of unsatisfactory living conditions. Micronutrient deficiencies are known as hidden hunger. Children, pregnant women, and nursing mothers present a higher risk of vitamin A deficiency (BRASIL, 2009) [2].

The main animal food sources of retinol are liver, fish oils, eggs, milk, and dairy products (ALMEIDA-MURADIAN; PENTEADO, 2003) [5]. Several studies have shown that liver, especially chicken liver, is an excellent source of vitamin A. Santos *et al.* (2009) evaluated the concentration of retinol in the liver of two different strains of chicken, Cobb and Ross, subjected to the same breeding process, which presented retinol values of 6678.0 μg/100g and 8324.1 μg/100g, respectively. Rüegg and Dimenstein (2018) evaluated three batches of chicken liver from three brands sold in the supermarket, and found mean values of 9152.9 μg/100g, 4673.1 μg/100g, and 5943.6 μg/100g. The Dietary Reference Intakes (DRI) is a commonly used reference for daily nutritional recommendations. The daily recommendation of vitamin A is 700 μg for women over 14 years old and 900 μg for men over 14 years old (INSTITUTE OF MEDICINE, 2001) [6].

Thus, in addition to its low cost, chicken liver has great potential to combat VAD (HOWELLS; LIVESEY, 1998 [7]; JAŚKIEWICZ; SAGAN; PUZIO, 2014 [8]; KANG; CHERIAN; SIM, 2006 [9]; MAJCHRZAK; FABIAN; ELMADFA, 2006 [10]; SCHINDLER; SCHOLZ; FELDHEIM, 1987 [11]; SURAI; KUKLENKO, 2000 [12]; TORRES *et al.*, 1998 [13]).

Several studies have been conducted for the detection of retinol and retinyl

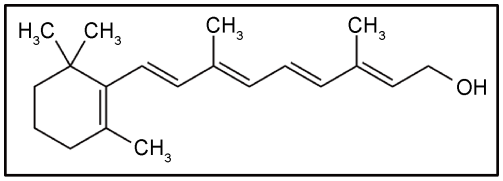


Figure 1. Chemical structure of retinol.

esters in animal food sources using liquid chromatography in reverse phase and UV absorption detection (FURR; COOPER; OLSON, 1986 [14]; KANE; FOLIAS; NAPOLI, 2008 [15]; KARADAS *et al.*, 2005 [16]; KIM; QUADRO, 2010 [17]; SATOMURA; KIMURA; ITOKAWA, 1992 [18]; SHINTAKU *et al.*, 1998 [19]; TATUM; CHOW, 2005 [20]). However, most of these methods involve the use of more than one solvent under gradient conditions, which increases the cost of analysis. None of these tests used a 100% methanol mobile phase in an isocratic elution process.

Our study aimed to develop and validate a rapid and cheap method to extract and detect retinol in chicken liver using high-performance liquid chromatography (HPLC).

2. Material and Methods

2.1. Reagents and Chemicals

The all-*trans* retinol standard was acquired from Sigma-Aldrich, with ≥ 95% purity. The standard was stored according to the label instructions.

HPLC grade methanol was purchased from J. T. Baker. Other reagents including absolute ethanol (99.8%) and ethanol (95%) were acquired from Sigma-Aldrich (Rio de Janeiro/RJ), and hexane was obtained from Merck S.A. (Cotia/SP).

2.2. Instrumentation

The HPLC instrument consisted of an LC-20 AT pump coupled to an SPD-20A UV-VIS detector and a Rheodyne injection valve with a 20 µL loop. A Hamilton syringe for manual injection with 100 µL capacity and a computer with the *LCsolution* software for data acquisition and processing were used.

2.3. Chromatographic Conditions

The chromatographic separation was performed with a reversed phase column (Luna 5u C18 (2) 100A Phenomenex®, 250 mm × 4.6 mm) kept at a temperature of 23˚C. The UV-VIS detector was set to monitor the retinol absorbance at 325 nm.

The mobile phase used for the analysis of retinol in the samples was methanol (100%) in an isocratic system with a flow of 1 mL/min.

Before use, the equipment was maintained under initial conditions with a flow rate of 1 mL/min of methanol (100%) for 30 min.

2.4. Stock and Working Solution Preparation

The retinol solution standard was prepared by weighing 1.0 mg of retinol (Sigma) in an Eppendorf tube, followed by dilution in 1.0 mL of absolute ethanol and stirring for 1 min. Then, two further dilutions were carried out to reach an approximate concentration of 10 µg/mL (**Figure 2**). A spectrophotometer (U-2000 model from Hitachi) and quartz cuvettes were used for absorbance measurement.

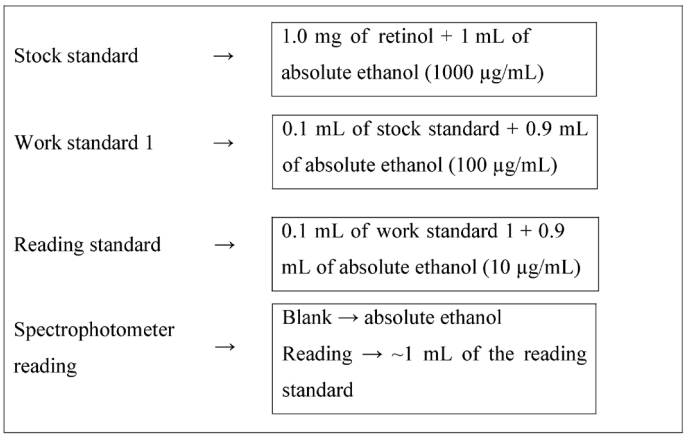


Figure 2. Methodology for the preparation of the reading standard.

The concentration of the standard was confirmed by the specific extinction coefficient of retinol in absolute ethanol (ε 1%, 1 cm in ethanol = 1780 at 325 nm) (MILNE; BOTNEN, 1986 [21]; SOLOMONS, 2012 [22]).

After confirmation of the actual concentration of the standard, a new dilution was performed from the reading standard in absolute ethanol (**Figure 3**), resulting in an approximate concentration of 1 µg/mL (1 ng/1µL) for use in HPLC.

2.5. Sample Preparation

For the analysis of retinol in frozen chicken liver, 1 kg of fresh chicken liver was purchased at a supermarket in Natal, Brazil. The chicken liver was placed in a blender (Arno model) together with one liter of saline solution (NaCl, 0.9%), forming a homogenate at 50%. Falcon tubes were filled with 1 g of the homogenate and stored on a freezer at −18˚C for 0, 1, 15, and 30 days.

2.5.1. Extraction of Retinol from Chicken Liver

The extraction of retinol from chicken liver was performed according to Hosotani and Kitagawa (2003) [23] and described below.

One milliliter of ethanol (95%) was added to each Falcon tube containing 1 g of the homogenate in order to precipitate the proteins present in the sample.

Before lipid extraction, saponification was achieved by adding 1 mL of potassium hydroxide (KOH, 50%) and the samples were placed in a water bath at 60˚C for 1 hour under stirring in order to hydrolyze the retinyl esters present in the samples.

For lipid extraction, the samples were washed with 2 mL of hexane and stirred for 1 min, and then were left standing for 5 min. The supernatant was then placed in a new tube, and this process was repeated twice, resulting in a final volume of approximately 6 mL of hexane. An aliquot of 250 µL of hexane was taken, placed in another 5 mL-polypropylene tube and evaporated to dryness in a water bath at 37˚C under nitrogen atmosphere (*White Martins*®). Aliquot was due to two reasons: evaporation time of hexane and the aliquot prevents HPLC

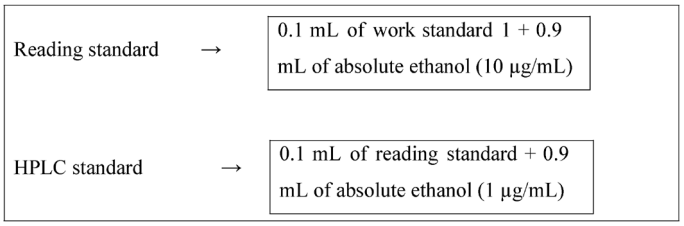


Figure 3. Methodology for the preparation of the HPLC standard.

column obstruction due to the high content of retinol in chicken liver samples. The samples were then stored at −18˚C under nitrogen atmosphere, and protected from light. Before HPLC processing, the samples were redissolved in 1 mL of absolute ethanol, and 20 µL of the samples was then used for HPLC. The measurements were carried out in duplicate injections and the average was calculated over the two obtained areas.

2.5.2. Retinol Identification

The identification and quantification of retinol in the samples was evaluated by means of comparison of the retention time of the all-*trans* retinol standard and the peak in the sample analyzed under the same conditions comparison of the areas in the chromatographic profile with the areas of the pattern.

2.5.3. Validation

The validation of the method was performed according to the current FDA guidelines, *i.e.*, the Guidelines on Bioanalytical Method Validation (FOOD AND DRUG ADMINISTRATION, 2013 [24]).

2.5.4. Linearity

The linearity of the method is the instrumental response of the linear calibration relationship (obtained area) with the concentration of the standard, being satisfactory when the correlation coefficient is close to 1 (BRITO *et al.*, 2003) [25].

Six different dilutions of the retinol standard were used for application in HPLC. Next, the plot of the linear equation was built from their respective areas, and a straight line equation was obtained through linear regression (peak area *versus* concentration of the standard).

2.5.5. Stability

The stability of the retinol stock standard was determined in triplicate with aliquots that were stored in a freezer at −18˚C and evaluated in cycles of 1, 15, and 30 days.

In order to evaluate the stability of retinol in the chicken liver samples, the latter were stored in a freezer at −18˚C and assessed in thawing cycles at room temperature (24˚C) for 8 hours after 1, 15, and 30 days.

2.5.6. Precision

The precision of the method was analyzed through a repeatability test. For the

assessment of the intra-precision, five replicates at three different retinol concentrations in the range of 2.0 - 5.0 μg/mL were prepared and analyzed in the same day.

For the assessment of the inter-precision, the intra-precision measurement was repeated for five consecutive days. The coefficient of variation (CV) was calculated from the standard deviation of the mean and expressed as a percentage.

2.5.7. Accuracy

The accuracy of the method was evaluated by a recovery test. Known amounts of the retinol standard solution were added in triplicate at three different concentrations (0.00436, 0.008072, and 0.012108 mg/mL) to the chicken liver samples. These concentrations were a practical application in HPLC and it was set to identify peaks at this concentration. These samples were subjected to the same retinol extraction method and analyzed by HPLC. The recovery was calculated by the ratio of the sample peak area and the standard solution peak area added 100 times, and expressed as a percentage.

2.5.8. Limit of Detection (LOD) and Limit of Quantification (LOQ)

In order to determine the LOD and LOQ, several injections were performed at decreasing concentrations. The lowest detectable, but not quantifiable concentration is the LOD. This in turn was detected by a visual method using a matrix after addition of known concentrations so that the analytical signal was distinguished from the noise for the lowest detectable concentration (RIBANI *et al.*, 2004) [26].

The lowest concentration that can be quantified with acceptable precision and accuracy is the LOQ. This was detected when the analytical signal was identified in the lowest equivalent dilution (RIBANI *et al.*, 2004) [26].

3. Results and Discussion

The use of HPLC has become a common method for the quantification of vitamins and minerals because of its sensitivity and accuracy. The development of methods based on HPLC presents many advantages such as speed, resolution power, the reduction of the amount of sample and solvent used, and low cost; thus encompassing several environmental benefits (CHEN; KORD, 2009) [27].

3.1. Linearity

Six different dilutions of the retinol standard (3.37 ng/20μL, 6.75 ng/20μL, 13.49 ng/20μL, 26.98 ng/20μL, 53.96 ng/20μL, and 107.92 ng/20μL) were applied for HPLC. The linear plot was built from their respective areas (26.426, 55.687, 119.135, 238.471, 498.955, and 989.117). The equation of the straight line obtained by linear regression, with R^2 = 0.9999, is shown in **Figure 4**, illustrating the linear relationship of peak area *versus* concentration of the standard.

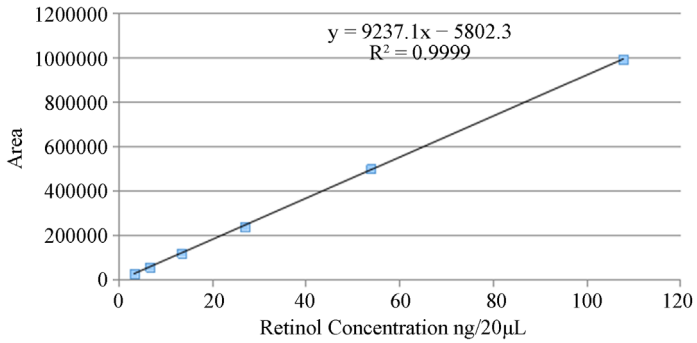


Figure 4. Calibration curve and linear equation obtained by analyzing different concentrations of the retinol standard with HPLC.

3.2. Stability

The retinol stock solution standard was stable for at least 30 days. The area of the standard peak was compared to the value of the area of the peak in the chromatogram measured on a particular day, yielding a stable average value of 93.2%.

The stability of retinol in the freeze–thawing process of chicken liver samples was determined to be at least 30 days, yielding a stable average value of 97.5%.

3.3. Precision

The intra- and inter-precision data for the method are shown in **Table 1**. Values below 15% are considered acceptable (FDA - FOOD AND DRUG ADMINISTRATION, 2013) [24]. As it can be seen, the precision values of the present method were all found to be below 15%.

3.4. Accuracy

The accuracy of the method was assessed through the recovery of the standard added to chicken liver samples at three different concentrations. The results are summarized in **Table 2**.

The average recovery ranged between 93% and 101.2%, while the coefficient of variation for the recovery ranged between 0.36 and 0.98. The results indicate that the method is precise for the determination of retinol in chicken liver.

3.5. LOD and LOQ

The limit of detection and limit of quantification are shown in **Table 3**. The values of sensitivity are satisfactory for the precise determination of retinol in chicken liver.

3.6. Applicability of the Method

The applicability of the method was verified using liver samples subjected to retinol extraction. A stock standard solution of retinol (with a concentration of 1217 ng/mL) was added to one of the samples (what is called '*spiking*') to assess

Table 1. Intra- and inter-precision of the method for the determination of retinol (CV: coefficient of variation).

Analyte	Intra-precision		Inter-precision	
	μg/mL	CV (%)	μg/mL	CV (%)
Retinol	2.0	3.0	2.0	9.7
	4.0	2.3	4.0	14.2
	5.0	7.8	5.0	12.2

Table 2. Recovery values of the method for the determination of retinol.

Concentration of the standard added (ng/mL)	Recovery (%)	CV (%)
4036	93	0.98
8072	98.5	0.36
12 108	101.2	0.91

Table 3. Limit of detection (LOD) and limit of quantification (LOQ) of retinol.

Parameter	Concentration (ng/mL)
LOD	9.1
LOQ	18.26

the suitability of the method and peak separation. **Figure 5** and **Figure 6** show the resulting chromatograms, where the successful separation of the analytes can be observed.

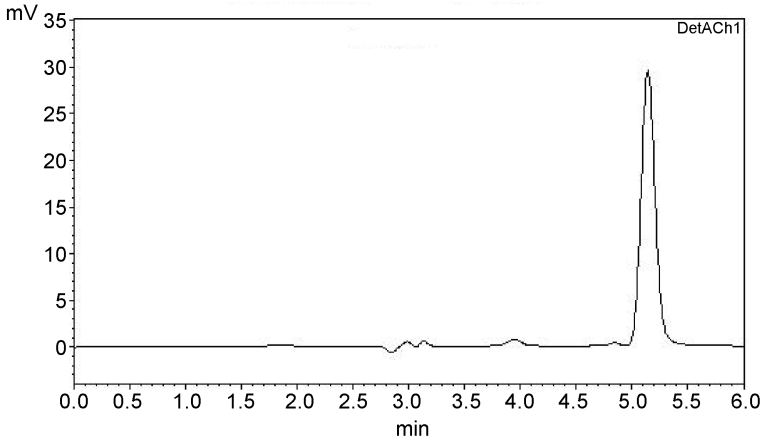
Other experimental applicability was made. Samples from organic, free-range and three brands of chicken liver, chilled, within the expiry date, and traded in supermarkets in the greater area of Natal, Brazil, were evaluated by using this method. This study found statistically different values among the means of the three different chicken liver (RÜEGG; DIMENSTEIN, 2018) [28].

Rüegg and Dimenstein (2018) also used this method to evaluate retinol from chicken liver in different cooking and defrost methods. The authors found a statistically decreased when chicken liver was submitted for 1 min in a microwave in defrost function.

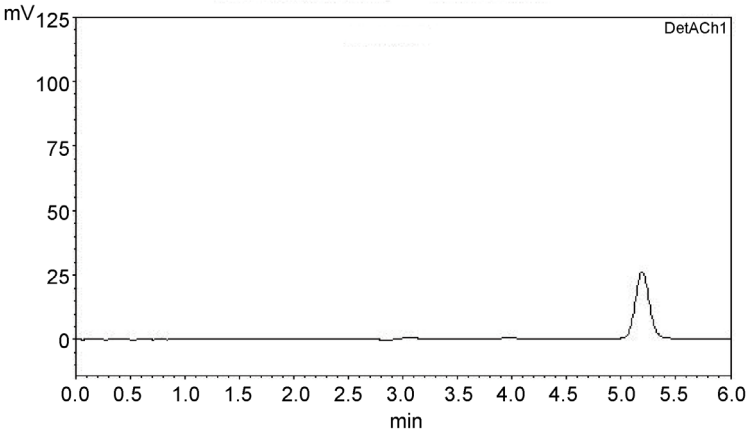
3.7. Comparison of the Method

The present study corroborates with other methodologies using methods similar to this one developed with liver of chicken.

Yokota and Oshio (2018) [29] developed a methodology for extraction, identification and quantification of retinol in mice's livers, also using 100% methanol in the mobile phase, obtaining a mean retention time of 3.2 minutes using a Mightysil RP-18 GP column. A similar result was observed with de Aquino *et al.* (2006) [30], which had a mean retention time of 4.3 minutes, using LC Shim-pack CLC-ODS (M) 4.6 mm × 25 cm column and the same mobile phase but in bovine liver.



(a)



(b)

Figure 5. HPLC elution profile of retinol. (a) All-*trans* retinol chromatogram of a 24.35 ng/20μL sample. (b) Chromatogram of a chicken liver sample subjected to retinol extraction.

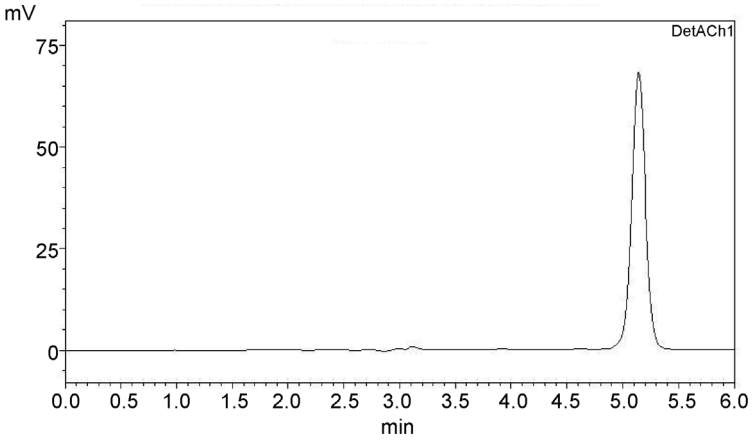


Figure 6. Chromatogram of a spiked sample of chicken liver with an all-*trans* retinol standard (1217 ng/mL).

Other methodologies using a gradient elution are also seen in the literature, for example combinations of methanol and acetonitrile (15:85 v/v) in the identi-

fication and quantification of retinol in pig liver, obtaining an average retention time of 4 minutes (MAJCHRZAK; FABIAN; ELMADFA, 2006) [10].

A mean retention time of about 3 minutes was observed in the study of Howells and Livesey (1998) [7] with pigs, cattle and chickens livers and using acetonitrile, n-butanol and acetic acid (60:40:0.1 v/v/v) as the mobile phase, but in a flow of 1.5 mL/min and a C18 column, different from the present study that used a flow of 1.0 mL/min and a Luna 5u C18 (2) 100A Phenomenex® column.

4. Conclusions

Although the retention times changed depending on column batch, flow speed, and consistency of the mobile phase, it was possible to identify and quantify the amount of retinol in chicken liver samples using reverse phase HPLC coupled with UV-VIS detection of its absorbance at 325 nm using an isocratic method with a single mobile phase (methanol 100%), resulting in an average retention time of 5.2 minutes.

The analytical method developed in this study is consistent with its function and with the current legislation policy, being economical, quick, easy, and suitable for its proposal.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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