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Validation of analytical method for monitoring of vitamin A in milks from *Programa Viva Leite*

Validação de método analítico para o monitoramento de vitamina A em leites do Programa Viva Leite

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ABSTRACT

Introduction: There is an evidence of hypovitaminosis A in certain populations. In order to combat this deficiency in Sao Paulo State, a government program was created for free distribution of pasteurized milk enriched with vitamins A, D, and iron for the low income population for the purpose to offer a food supplement with high nutritional value. **Objective:** Optimization and validation of a methodology for the determination of vitamin A in fluid milk, using modified methodology of Association of Official Analytical Chemists (AOAC). **Method:** The high performance liquid chromatography with fluorescence detection was used to evaluate vitamin A contents in 261 milks of the program. **Results:** The validated analytical method was adequate for the determination of vitamin A in fluid milks in the laboratory routine. The results showed that 52% of the samples had vitamin A concentrations above the declared value in the nutrition facts label, while 11% presented lower content in comparison to the declared value. **Conclusions:** Monitoring of vitamin A levels in these milks should be continuous to ensure the amount of micronutrient declared on the label and to meet the objectives of the program.

KEYWORDS: Vitamin A; Validation; Milk; Governmental Program

RESUMO

Introdução: A ocorrência de hipovitaminose A é evidente em determinadas populações. Com o intuito de combater essa deficiência no estado de São Paulo, foi criado um programa governamental com distribuição gratuita de leite pasteurizado enriquecido com vitaminas A, D e ferro para a população de baixa renda, com a finalidade de oferecer um complemento alimentar de alto valor nutritivo. **Objetivo:** Otimizar e validar uma metodologia analítica para determinação de vitamina A em leites fluidos, utilizando metodologia oficial da *Association of Official Analytical Chemistry* (AOAC) com modificações. **Método:** Foi utilizada a cromatografia líquida de alta eficiência com detecção por fluorescência para avaliar os teores de vitamina A em 261 amostras de leites distribuídos pelo programa. **Resultados:** O método analítico validado se mostrou adequado para a determinação de vitamina A em leites fluidos na rotina do laboratório. Os resultados indicaram que 52% das amostras apresentaram concentrações de vitamina A acima do valor declarado na informação nutricional da rotulagem, enquanto 11% apresentaram teores abaixo do valor declarado. **Conclusões:** O monitoramento dos teores de vitamina A nestes leites deve ser contínuo para garantir a quantidade de micronutriente declarada no rótulo e atender os objetivos do programa.

PALAVRAS-CHAVE: Vitamina A; Validação; Leite; Programa Governamental

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INTRODUCTION

Vitamin A is an essential nutrient for the normal functioning of vision, growth, development and maintenance of epithelial cell integrity, immune function and reproduction[1].

According to the Brazilian Ministry of Health (MS), the recommended daily intake (RDI) of vitamin A is 600 µg for adults, between 375 µg and 500 µg for babies and children, according to their age, and 800 µg and 850 µg, respectively, for pregnant and nursing women[2].

Vitamin A deficiency is considered to be one of the major nutritional deficiencies in underdeveloped countries and is the leading cause of preventable blindness in the world. It is also associated with 23% of deaths caused by diarrhea in children[3]. In Brazil, the infant population of the Northeast is the most vulnerable to the problem, but there are indications of occurrence of hypovitaminosis A also in areas of abject poverty in the Southeast region[4,5]. In order to reduce this deficiency, in 1999 the government of the state of São Paulo created the Viva Leite Program for the free distribution of pasteurized milk enriched with iron and vitamins A and D for the low-income population. The program provides 75 million liters of milk for children and elderly people in situations of food insecurity and social vulnerability and currently serves about 420 thousand families[6].

The World Health Organization recommends exclusive breastfeeding up to 6 months of age; after this stage, additional food should be included, and breastfeeding should be maintained until at least 2 years of age. Breast milk contains all the proteins, sugars, fats, vitamins, and water that baby needs to be healthy[7]; moreover, it presents certain antibodies and white blood cells that offer immunity in this period. In adulthood, the consumption of cow's milk provides some of these essential nutrients, supplying the body with energy, high quality proteins and a variety of vitamins and minerals. Nevertheless, thermal processing can cause nutritional losses, especially of vitamins[8].

Enriching milk with vitamins is becoming an increasingly common practice and can be applied either to compensate for nutritional losses from processing or to increase its nutritional value. The process of milk enrichment must be well controlled since the vitamin A can be easily degraded, because it is photo- and heat sensitive, easily oxidized and unstable at pH below 4.5. Thus, for food fortification, vitamins in the ester forms, such as acetate or retinyl palmitate, are most commonly used because they are more stable with respect to their free form[9].

The standardization of analytical methodology for the quantification of vitamins in food is difficult due to the diversity of procedures described in the literature and to the complexity of the existing matrices. Nowadays the most used techniques are high performance liquid chromatography (HPLC) with ultraviolet/visible (UV-VIS) detectors, diode array (DAD) and/or fluorescence (FLU) detectors, with normal or reverse phases[10,11]. In recent years, the development of techniques such as ultra-high performance liquid chromatography combined with the mass spectrometer has presented several advantages such as reduced analysis time, lower solvent utilization and higher efficiency. However, for most public laboratories, it is still a costly technique[12].

The analysis of the vitamin A levels in the milk distributed by the Program is extremely important to verify if the improvement plants are properly enriching the product. However, for the results to be reliable, the analytical method must be validated by an official standard such as that established by the National Institute of Metrology, Quality and Technology (Inmetro)[13].

The objectives of this study were to present the optimization and validation steps of the methodology for the determination of vitamin A in fluid milk by HPLC-FLU, as well as to apply the method for the evaluation of enriched milk samples of the Viva Leite Program.

METHOD

Samples

Samples of whole pasteurized milk and ultra high temperature (UHT) skim milk used in the validation of the method were purchased from local stores in São Paulo city.

The Viva Leite Program was executed by the Sanitary Surveillance body of São Paulo state in 261 samples collected in 12 municipalities. Vitamin A results obtained in the analyses were compared with the values declared in the nutrition facts of the label.

Standards and reagents

For the validation of the methodology, the standards used are all-trans-retinol and retinyl palmitate, by Sigma-Aldrich (St. Louis, USA), and the following reagents (PA grade): petroleum ether and ethyl alcohol (96%), by Synth (Rio de Janeiro, Brazil); potassium hydroxide (KOH), ascorbic acid, pyrogallol and butyl hydroxy toluene (BHT), by Merck (Darmstadt, Germany). Methanol and isopropanol, both of chromatographic grade, were obtained from the Carlo Erba (Milan, Italy).

Methodology optimization

First the chromatographic conditions of separation and detection of the all-trans-retinol standard by HPLC-FLU were assessed. The method of the Association of Official Analytical Chemists (AOAC)[14] establishes the normal phase liquid chromatography system, but the reverse phase system was chosen because it required less toxic solvents. Thus, mobile phases composed of methanol with different proportions of water were tested until a peak with good resolution was obtained. Saponification conditions were also optimized: with different temperatures and saponification times in the attempt to reduce the time established by the AOAC method (18 hours). The extraction process was also optimized, using the smaller-volume samples and, consequently, generated a smaller amount of organic solvents in relation to the AOAC method. Additionally, the use of BHT, pyrogallol and ascorbic acid antioxidants was investigated in order to select the most efficient option, considering the

percentage of recovery of the all-trans-retinol standard added to the whole pasteurized milk sample.

Proposed methodology

The methodology was based on that described by AOAC[14], with the modifications described above, according to the following steps:

Saponification and extraction: In polyethylene tubes with 25 mL capacity, 2 mL of sample, 3 mL of KOH (3.8 mol.L^{-1}), 2 mL of ethyl alcohol and 1 mL of BHT (0.1% in ethyl alcohol) were added. The tubes were agitated for 2 min in a vortex type stirrer (model QL-901, Biomixer, Ribeirão Preto, Brazil) and then rested for 16 hours under light for complete saponification of the esters. After saponification, 10 mL of petroleum ether, and 10 mL of water were added, and stirred it for 30 s in a vortex stirrer; after stirring, 1 mL of ethanol was added. The tubes were centrifuged at 2500 rpm for 10 min (NT 812 model centrifuge, Novatécnica, Piracicaba, Brazil), the ether phase was transferred to a glass vial with a micropipette and the extraction process was repeated twice, except for the addition of water. The solvent was completely evaporated in a sample concentrator (model TE-019-E3, Tecnal, São Paulo, Brazil) with maximum heating up to 45° C under nitrogen flow. The samples were resuspended in 1 ml of methanol, filtered on 0.45 µm PTFE membranes (Millipore Corp., Bedford, MA, USA), transferred to an amber vial and analyzed on the same day by HPLC.

Chromatographic analysis: The determination of vitamin A was performed on Shimadzu liquid chromatograph (Kyoto, Japan), composed of LC-20AT pump, CBM-20A controller, CTO-20A column oven and RF-10AXL fluorescence detector (excitation wavelength of 325 nm and emission of 480 nm). A LiChrospher 5 RP18 reverse phase column (250 mm x 4.6 mm, 5 µm particles) was used with LiChrospher 5 RP18 guard column (25 mm x 4.6 mm, 5 µm particles), Varian brand (Palo Alto, USA); as the mobile phase, 100% methanol was used, with a flow of 1 mL.min^{-1} in isocratic mode; the injection volume was 20 µL and the oven temperature (column) was 28° C.

A stock solution of the standard was prepared at the approximate concentration of 5,000 ng.mL^{-1} in isopropanol; the correction of the concentration value was performed by spectrometry analysis with absorbance at 324.5 nm (UV/Vis spectrophotometer model 8453, Hewlett Packard, Palo Alto, USA), using the Lambert-Beer Law, represented by the following formula:

$$A = \varepsilon.b.c$$

Where:

A = absorbance of vitamin A at the wavelength of 324.5 nm
ε = molar absorptivity of vitamin A (ε = 5,460 L.mol^{-1}.cm^{-1})[14]
b = optical path (1 cm)
c = molar concentration of vitamin A in the solution (mol.L^{-1})

Methodology validation

The methodology was validated according to the DOQ-CGCRE-008 document - Guidance on validation of analytical methods, from the General Coordination of Accreditation, Inmetro[13]. The following performance parameters were determined:

Selectivity: Samples of pasteurized milk and skim UHT milk were fortified with all-trans-retinol standard at three concentration levels: 594.3; 1,816.4; 3,527.3 ng.mL^{-1} (pasteurized milk) and 284.3; 1,137.1; 3,411.5 ng.mL^{-1} (UHT milk). The results obtained from the fortified matrices were compared with the standard solution of all-trans-retinol prepared in methanol. The analytical curves of the three groups were plotted and the matrices effects of the pasteurized milk and skim UHT milk were verified by visual comparison of the slope of the curves and by Student's t-test.

Linearity and working range: The linearity study was performed with six concentration levels, between 200 and 5,000 ng.mL^{-1}, using all-trans-retinol as the standard. The standard solutions were prepared in methanol, in triplicate, to obtain the linear regression equation by least squares method. The Grubbs test was applied at each level to verify aberrant values and the Cochran test was used to evaluate the homogeneity of the variances or homoscedastici behavior of the residues.

Limits of detection (LOD) and quantification (LOQ): The first point of the calibration curve was established as the limit of quantification. Once LOQ was established, this value was confirmed by the analysis of independent samples at the same concentration level with six replicates of skim UHT milk fortified with all-trans-retinol standard. The Grubbs test was applied to evaluate aberrant results. LD was established from LOQ through the formula: LOD = LOQ/3.3.

Accuracy: Accuracy was assessed by the recovery test of the added standards in the matrices of pasteurized whole milk and skim UHT milk. The analyses were performed in triplicate with the all-trans-retinol standard, at three levels of concentration: 594.3; 1,816.4; 3,527.3 ng.mL^{-1}(pasteurized milk) and 284.3; 1,137.1; 3.411.5 ng.mL^{-1} (UHT milk). The acceptance criteria for recovery were values between 95% and 105%.

Precision: For repeatability, the results obtained in the accuracy test for fortified samples were used; the standard deviation for each concentration level and the relative standard deviation (RSD) were calculated. RSD values below 10% suggest the repeatability criterion.

RESULTS AND DISCUSSION

Methodology optimization

The optimization was initiated by the chromatographic conditions of separation and detection of the all-trans-retinol standard by HPLC-FLU. Initially, methanol: water (95:5, v/v) mobile phase was used, however, under these conditions, the retinol chromatographic peak showed a tail. When using 100% methanol as the mobile phase, the results were satisfactory, with good efficiency of the chromatographic peak (Figure 1). To maintain the efficiency of the column, a cleaning in the chromatographic system was performed weekly using a solution composed of methanol: acetonitrile: 2% acetic acid (35:35:30, v/v/v), with a flow of 1 mL.min^{-1} for 45 min.

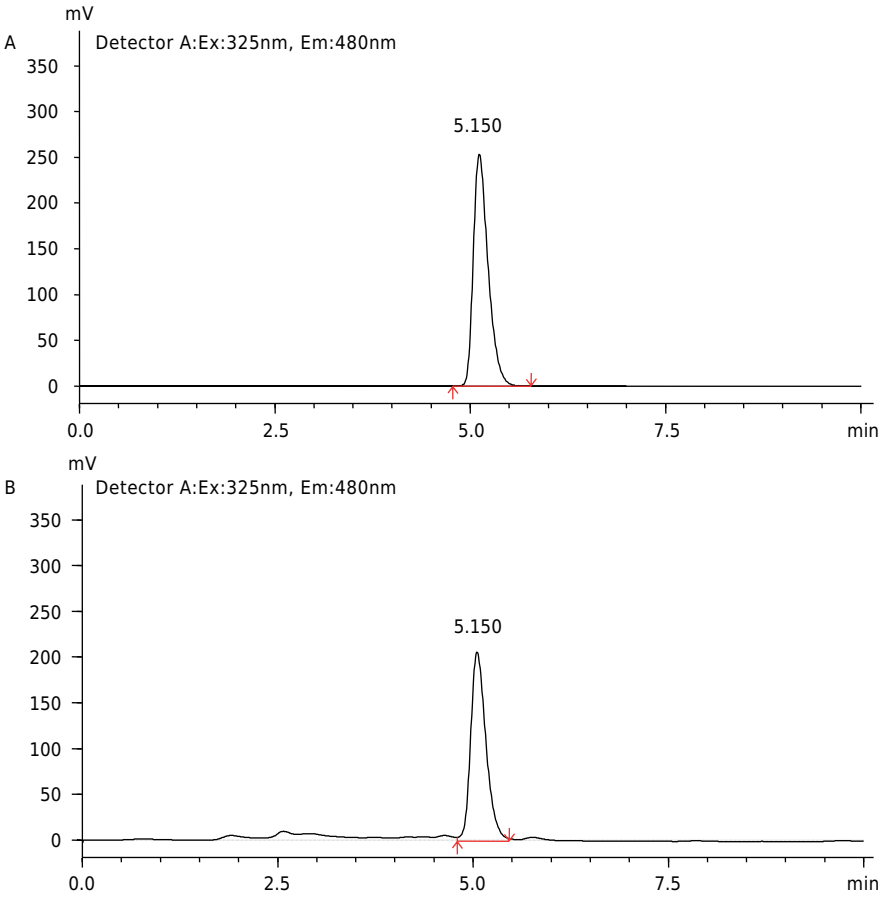

Figure 1. Chromatograms of the standard solution of all-trans-retinol at 1.800 ng.mL^{-1} in methanol (A) and the extract of pasteurized milk sample enriched with vitamin A in methanol (B). Chromatographic conditions: C18 column (250 mm x 4.6 mm, 5µm); mobile phase: 100% methanol; flow 1 mL.min^{-1}, in isocratic mode.

Extracting vitamin A from food usually requires an additional step of saponification prior to the extraction with an organic solvent. The saponification process enables the disruption of the ester bonds in the lipoprotein matrix, with release of fatty acids, glycerol, phospholipids and other molecules. Liposoluble vitamins such as vitamin A are found in unsaponifiable fractions and, with this procedure, the esterified forms of vitamin A are converted into free alcoholic forms, allowing quantification. On the other hand, degradation of these vitamins may occur, depending on the saponification conditions, or the presence of impurities in the solvents used in the extraction[15]. The method described in AOAC[14] recommends 18 hours at room temperature for the saponification of the vitamin A esters. Because of this relatively long time, attempts were made to reduce the time with an increase of the temperature: five different times (30, 45, 60, 90, and 120 min) were evaluated at 45° C, using samples of whole pasteurized milk enriched with retinyl palmitate standard. The vitamin A results were compared with the values obtained by saponification at room temperature for 16 hours. The greater the area of the chromatographic peak of retinyl palmitate, the lower the conversion of this to its free form, that is, the lower the efficiency of the established condition for saponification. Comparing the free retinol and retinyl palmitate chromatographic peaks areas, the saponified sample for 16 hours was the only that did the palmitate convert to the free form in a ratio greater than 99%.

The BHT, pyrogallol and ascorbic acid antioxidants were also evaluated by the analysis of the recovery of the all-trans-retinol standard in the sample of whole pasteurized milk, after 16 hours of saponification, and to verify the protective activity of vitamin A. The highest recovery was observed in the test using BHT (98%), followed by ascorbic acid (85%) and, finally, pyrogallol (80%). The use of ascorbic acid caused effervescence of the sample during agitation, favoring the loss of the analyte. Both ascorbic acid and pyrogallol did not offer adequate protection of vitamin A, probably because they presented water soluble characteristics[15].

Validation

Selectivity: Visual analysis of line slopes of the results obtained with the matrices (pasteurized milk and skim UHT milk) fortified with the all-trans-retinol standard indicated that there was apparently no matrix interference; this was confirmed statistically by the Student's t-test for the angular coefficients. The calculated t values ($t_{calc.\ UHT\ milk}$ = 0.362 and $t_{calc.\ pasteurized\ milk}$ = 1.014)

were lower than the tabulated ($t_{tab\ UHT\ and\ pasteurized\ milk}$ = 2.069), with 95% confidence interval.

As the results showed that matrix interference did not exist, calculations for linearity, LOD, LOQ, accuracy, and precision were performed using the calibration curve without the matrix.

Linearity and Working Range: At each concentration level, the Grubbs test was applied and no aberrant value was verified.

In the calibration curve, it was possible to evaluate the dispersion of the measurements as a function of the concentration; once the condition of variance is uniform, it is called homoscedasticity. To verify if the system is homocedastic (equal variances) or heterocedastic (different variances), the Cochran test was applied. The value of C_{calc} was 0.50 for a C_{tab} of 0.61 (triplicate at six concentration levels, with 95% confidence), confirming that the system is homocedastic, that is, it has similar variances along the working range. The residue graph of the calibration analytical curve presented random distribution, free of trends.

The coefficient of determination (R^2) provided an indication of how much the straight line can be considered as a mathematical model, since the value found was 0.9997, close to 1, indicating that the method was linear within the working range.

LOD and LOQ: The first point of the calibration curve (215.0 ng.mL^{-1}) was established as the limit of quantification. The results of the analyses of the six skim UHT milk replicates fortified with all-trans-retinol standard at this concentration level did not present aberrant values, since, in the Grubbs test, calculated G values (1.685 and 1.045) were lower than the tabulated (2.126, with 95% confidence). The RSD was less than 10%, indicating that the method has adequate accuracy, that is, it is repeatable. The limit of detection was calculated by the formula LOD = LOQ/3.3, resulting in a value of 65.1 ng.mL^{-1}.

Accuracy: The results of the vitamin A recovery assays added in the matrices are shown in the Table, and the recovery percentages of the addition of all-trans-retinol at all levels were within the established criteria, with values between 95% and 110%, indicating adequate recovery.

Table. Recovery and relative standard deviation (RSD) of vitamin A in pasteurized whole milk and ultra high temperature (UHT) skim milk.

Matrix (Vitamin A in ng.mL^{-1})	Added standard (ng.mL^{-1})	Recovery (%)	RSD (%)
Whole pasteurized milk (768.9)*	594.3	101.1	5.2
	1,816.4	100.3	5.6
	3,527.3	104.5	5.1
UHT skim milk (53.7)*	284.3	107.9	2.4
	1,137.1	105.3	3.3
	3,411.5	100.2	6.2

* Vitamin A concentrations analyzed in matrices without the addition of standard; analyses performed in triplicate at each level of fortification, with the addition of all-trans-retinol standard.

Precision: For the study of repeatability, the results obtained in the fortified sample recovery test were used, calculating the standard deviation for each concentration level and the relative standard deviation. The method shows the criterion of repeatability established throughout the working range, with RSD lower than 10% (Table).

Analysis of vitamin A in enriched milk

The vitamin A content declared in the nutrition labeling information of pasteurized milk samples is 120 µg equivalents of retinol (ER) in a 200 ml serving, equal to one cup. According to RDC Resolution No 360, of December 23, 2003, of the Brazilian Sanitary Surveillance Agency/MS (Anvisa/MS), referring to the nutritional information of packaged foods, a tolerance of 20% is allowed in relation to the values declared in the label, rendering acceptable analytical vitamin A results between 96 and 144 µg ER in the serving[16]. Figure 2 represents the percentages of the samples with vitamin A content in compliance, above and below the declared values in the labeling nutrition information.

Of the 261 samples analyzed, 136 (52%) had vitamin A contents above the declared value, and in 51% of them the values were between 144 and 200 µg ER, in 48% between 200 and 300 µg ER and one sample presented content above 300 µg ER in the serving.

A significant number of samples shows vitamin A levels above the declared value, which probably occurs because milk already naturally contains a certain amount of this vitamin. According to the Brazilian Food Composition Table of the University of São Paulo (TBCA-USP)[17], the concentration of vitamin A in pasteurized whole milk is between 42 and 54 µg ER in 100 mL. These concentrations may vary according to the diet, breed and genetic quality of the animals, in addition to the environmental conditions in which the animals live[18]. Many processing plants are likely to

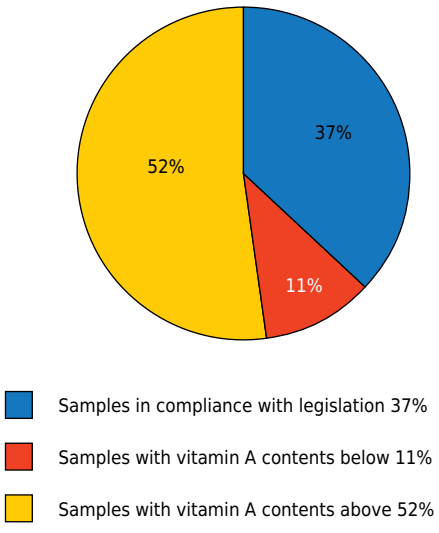

Figure 2. Percentages of analyzed pasteurized milk samples, with vitamin A content compliance, above and below the declared values in the label nutritional information.

ignore the amount of pre-existing vitamin in the milk and add the micronutrient in order to obtain a value close to that stated on the label.

RDC Resolution No 360/2003 of Anvisa/MS provides that, for products containing micronutrients in excess of the tolerance of 20%, the responsible company shall maintain studies that warrant such variation[16]. The same observation regarding overdose of micronutrients is verified in Ordinance No 31, of January 13, 1998, from the Department of Health Surveillance (SVS) of the Ministry of Health: Technical Regulation on Food Added with Essential Nutrients[19]. Thus, an overdose of vitamin A would be allowed, as it is susceptible to degradation when exposed to light, high temperatures and oxygen[15]. This overdose of vitamin A found in the analyzed samples is likely to guarantee the declared micronutrient concentrations up to the final product shelf life, assuming its possible degradation.

RDC Resolution No 269, of September 22, 2005, by Anvisa/MS, establishes the values of recommended daily intake considering the need to guide consumers and food producers on the recommended values of proteins, vitamins and minerals[2]. The RDI corresponds to the number of nutrients to be consumed daily to meet the nutritional needs of most individuals and groups of people of a healthy population and it was established on the basis of references from the Food and Agriculture Organization of the United Nations (FAO) and the Institute of Medicine (IOM)[2,20,21]. In addition to the recommendations for intake, the IOM also establishes the tolerable upper intake level (UL) for some micronutrients. UL is the highest daily intake of a nutrient that apparently does not pose a risk of adverse health effects for most individuals in a given group[21].

Vitamin A levels above the informed value observed in these milk samples are unlikely to pose health risks as the tolerable upper limit of vitamin A is 3,000 µg ER per day for a healthy adult, according to the IOM[21]. This limiting dose of vitamin A would hardly be reached with the consumption of pasteurized milk, since the population that benefits from this Program is low income, in most cases, without access to a nutritionally adequate diet.

The amount of vitamin A established for this program's milk (120 µg ER per serving) corresponds to 20% of the vitamin A RDI for a healthy adult and about 24% for children[19]. The purpose of this program is to provide nutritious food for a population prone to malnutrition, so vitamin A should be added to the milk in the minimum amount established.

For the samples that presented vitamin A concentrations below the declared value, the lowest value found was 63.0 µg ER in the serving. Low levels of vitamin A may be due to the lack of homogenization during the incorporation process of the nutrients, or the absence of enrichment of the product, or even its degradation.

CONCLUSIONS

The optimized and validated analytical method established for the determination of vitamin A in fluid milk proved to have selectivity, precision and accuracy, enabling to obtain reliable results in the routine laboratory analyses.

The application of the method in the analysis of enriched pasteurized milk indicated that 52% of the samples had an overdose of vitamin A, however, these levels do not represent risks to the health of the consumer, considering that this milk is distributed to a low income population that is prone to malnutrition.

The results of the vitamin A analyses below the declared value observed in the samples may indicate no enrichment or lack of control during the process. The lack of enrichment by the plants is an alarming fact and should be treated with attention by the Sanitary Surveillance agencies, as this affects the objective of the government program, which aims to reduce vitamin A deficiency in the poor population.

Vitamin A is essential to the immune system, the vision and the maintenance of cellular functions. Therefore, the monitoring of the levels of this vitamin in enriched milk should be constant, including corrective actions to repair any flaws in the enrichment process and ensure the provision of a suitable product for the population included in the Program.

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.