Analysis of Vitamin A in Jordanian Local Fresh Milk Samples Using Liquid Chromatography Tandem-Mass Spectrometry, LC/MS/MS

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Abstract- An auspicious method for the quantitative determination of vitamin A (retinol) was augmented in seven different Jordanian local fresh milk samples using liquid chromatography tandem -mass spectrometry (LC/MS/MS). The compound was separated with C18 Thermo fisher gold (4.50 x100 mm x 5 Å) column through a flow rate of 1 mL/min with isocratic mobile phase. Vitamin A acetate was used as an internal standard .The method was validated using triplicate analyses, relative recovery experiment and statistical analysis. Liquid-liquid extraction was employed as a pre-concentration step with n-hexane -dichloromethane mixture (90%:10%) as an extraction solvent. The concentration range of the working standards was 0.0-1000.0 ng/mL. The correlation coefficient was found to be 0.9994. The limit 0f detection and limit of quantification were found to be 0.53 and 1.78 respectively. The 95% confidence interval was calculated to be $49.03_2 \pm 0.22$. This technique showed reasonable recovery of 98.08%, coefficient of variation of 0.36%, standard deviation of 0.178 and error of -48.032. The ranges of the calculated concentrations of the vitamin A were found to be from 6.47 ng/mL- 46.35 ng/mL.

Index Terms- Analysis, Vitamin A, Fresh Milk, LC/MS/MS.

I. Introduction

Most people think that the only important sources of vitamins are fruits and vegetables. However dairy products are also important sources of vitamins more importantly fat soluble vitamins. Dairy products comprise many types of nourishment that are important for good health and nutrition. In infants and children, milk and milk products possibly supply a substantial fraction of vitamin A.

A vitamin is an organic compound required by an organism as a vital nutrient in limited amounts [1]. Vitamins generally act as catalysts, reacting with proteins to create metabolically active enzymes that in turn produce thousands of significant chemical reactions throughout the body [2]. Fat soluble vitamins are substances which are vital to the human health and they assist the body various processes [3]. Vitamin A, Carotenoid, Provitamin A, Vitamins D, E and K are the main classes of fat soluble vitamins [4].

A group of nutritionally unsaturated hydrocarbons, which include retinol, retinal, retinoic acid, and several pro-vitamin A carotenoids, among which beta-carotene is the most important, is termed vitamin A [5]. It has multiple functions: it is a significant tool for growth and development, for the maintenance of the immune system and good vision [6]. It is required by the retina of the eye in the form of retinal, which biologically joins with protein opsin to form rhodopsin, the light-absorbing molecule [7]. Treatment for Cancer, HIV, and Dermatological purposes have presently been established by pharmaceutics utilizing mega doses of naturally occurring retinoic acid derivatives [8]. Vitamins A, D, E, and K has been analyzed in breast milk, fortified and other foods [9], infant formula [10] and human blood serum by HPLC–MS/MS [11].

The major methods for the detection of fat soluble vitamins nowadays have been reported to be high performance liquid chromatographic ultraviolet/visible (HPLC–UV/Vis) and fluorescence (HPLC–Fl) detection [12] and gas chromatography [4]. Due to their chemical diversity and varying levels within samples, the compounds are usually determined individually. UPLC–UV/V technique however, was reported for the simultaneous determination of all *trans*-retinol, α-tocopherol and β-carotene in milk [13]. For quantification of a wide range of non-volatile compounds at the parts-per-million (mg/kg) and parts-per-billion (μg/kg) levels, high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) is broadly accepted as the point of reference [14]. Pharmaceutics utilizing mega doses of naturally occurring retinoic acid derivatives are currently in use for cancer, HIV, and dermatological purposes [8]. As reported by [4] that the major extraction methods are saponification/ solvent extraction, supercritical fluid extraction, and direct solvent extraction. In this work, direct solvent extraction was used.

This paper report the vitamin A (retinol) composition of 7 Jordanian commercially local fresh milk samples using liquid chromatography tandem mass spectrometry, LC/MS/MS. All the samples were bought within main Market and Sheikh Khalil Yoghurt Shops in Irbid, Republic of Jordan,

II. Materials and Methods

Apparatus

A chromatographic system of model API 300 Applied Biosystem consisted of an Agilent stable-bond C18 Thermo gold column (4.50 x100 mm x 5 Å) with in-build detector was used. The guard and analytical columns were mounted in a thermo stated column compartment set at 30 °C. The peak areas were

integrated automatically by computer using Analyst software version 1.4 (AB Sciex) software program. Other apparatus used included a centrifuge, normal evaporator and vortex.

Chemicals used

Pure vitamin A and vitamin A acetate with analytical grade methanol, ethanol, hexane, dichloromethane, formic acid and acetonitrile , were purchased from Sigma –Aldrich Chemicals.

Procedure for high-performance liquid chromatography Chromatographic Conditions

Solutions and mobile phases were prepared at the time of use. The mobile phases used were methanol: acetonitrile: deionized water: formic acid (68:30:2:0.1 v/v/v/v) (pH 3.5). The analytical column used was C18 Thermo gold (4.50 x100 mm x 5 Å). All analysis was done under isocratic conditions at a flow-rate of 1.0 ml min^{-1} and at room temperature.

Standard Solutions

10 mg of pure vitamin A and vitamin A acetate standards were accurately weighed and transferred into two different 100-ml volumetric flasks. To each volumetric flask, 100 mL pure ethanol solution was added to form 100 mg/L stock solutions respectively. Further dilutions with two aliquots 100 µL from each of vitamin A and vitamin A acetate stock solutions in ethanol resulted in 100 ng/mL of vitamin A and vitamin A acetate solution respectively. Serial dilutions with 100 ng/mL of vitamin A acetate (internal standard) was done and the following working standard solutions (0-1000 ng/mL) were obtained.

Samples Preparation

1 g of each fresh milk samples was accurately weighed and transferred to a 10- mL plastic tube containing 1ml internal standard (vitamin A acetate) and 1mL of 39% ethanolic sodium hydroxide solution was added . The mixture was then heated in a water bath at 60^{o}C for 40 minutes. Extraction was done by adding 4 mL hexane-dichloromethane mixture (90%:10%) and shaked vigorously for 1 minute to yield two different layers (organic and aqueous layer). The hexane- dichloromethane extracts was decanted, washed with a further 2 mL of two aliquots of water. The hexane- dichloromethane extracts was then taken and evaporated to dryness. The residue was reconstituted with (300µL) of mobile phase((methanol(68%): acetonitrile(30%): water(2%) formic acid (0.1%)) and filtered before taken to LC/MS/MS analysis.

Several volumes of hexanes were also used for the extraction but a mixture of hexane- dichloromethane (90:10) was found to be the best extraction solvent for this analysis. Increasing volume of the internal standard from 100 µL to 300µL made the analysis to be more feasible. Also increasing injection volume from 50 µL to 100 µL improved the analytical resolution. Substitution of potassium hydroxide with sodium hydroxide made the reaction to be selective as potassium hydroxide gave a poor impact during sample preparation.

Calibration and linearity

Calibration curves were constructed in the range 0.0–1000.0 ng/mL to incorporates the expected concentrations in measured samples. Curves were obtained by plotting the peak area of these pure vitamins against concentrations of these vitamins. Linear calibration curves were generated by linear regression analysis and achieved over the corresponding standard concentrations ranges.

Analytical recovery

Absolute recoveries of 5 different concentrations of vitamin A (0–1000.0 ng/mL) in fresh milk samples were verified by examining the samples as described above and comparing the peak areas of both vitamins with those acquired from direct injection of the compounds dissolved in the processed blank sample.

Precision and accuracy

The precision and accuracy of the examination was determined based on analysis of quality control samples. Dairy products quality control sample concentrations for vitamin A were 0.0–1000.0 ng/mL. Five replicate quality control samples at each concentration were analyzed and the means, standard deviations (SD) and coefficients of variation (C.V.) were calculated by standard methods.

III. STUDY FINDINGS

Linearity, LOD, and LOQ

Standard solution progressions with a range of concentration of 0.00 to 1000 ng/mL were prepared by diluting the stock solutions in ethanol. The standard solutions were injected in triplicate. Peak areas were plotted against the concentration of the analyte injected, and linear regression equation was acquired. The linear ranges were ~ 0.00–1000 ng/mL and the correlation coefficient was found to be 0.9994. The results obtained for LOD and LOQ are listed in Table 1. The LOD was found to be 0.53 ng/mL where as the LOQ was found to be 1.78 ng/mL.

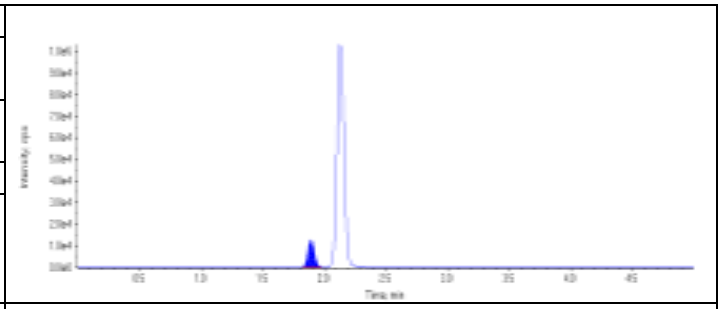
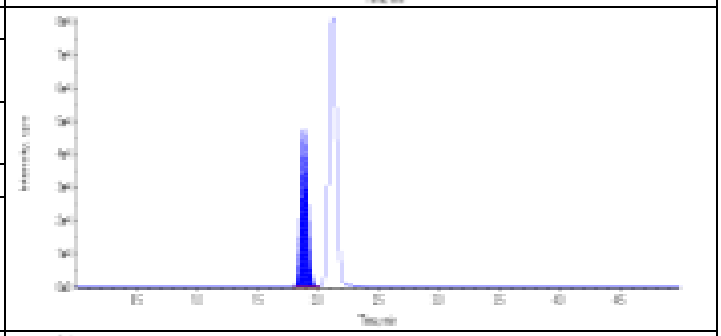
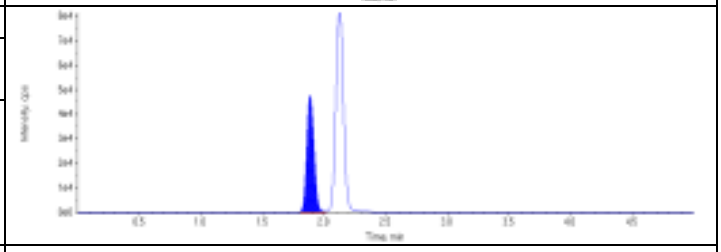
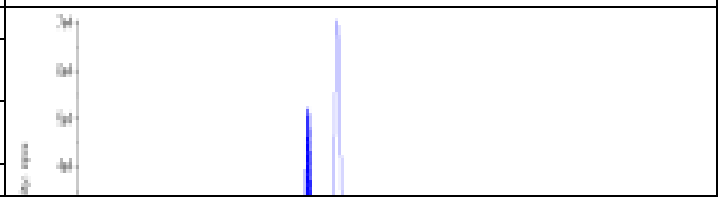
Table 1: Recoveries, CV, SD, LOD, LOQ, CV and Error Values Obtained from the Standard Addition Method in Local Fresh Milk Samples.

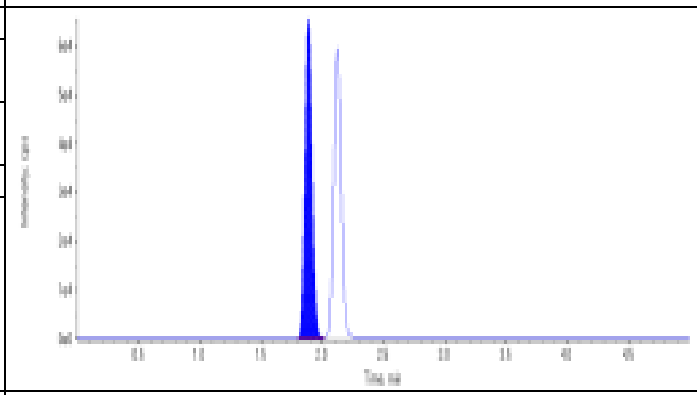
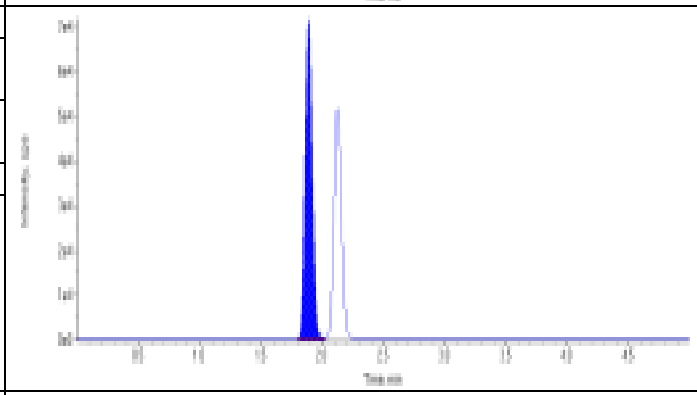
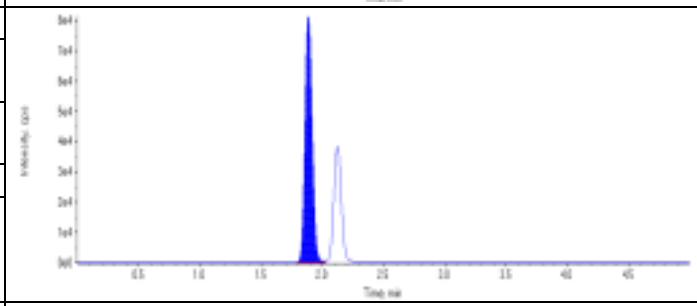
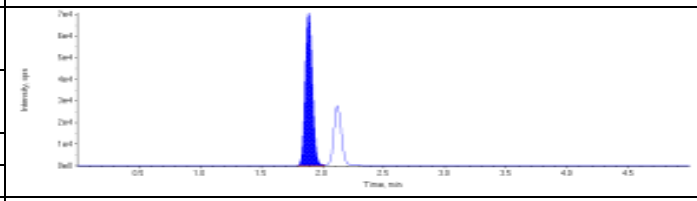
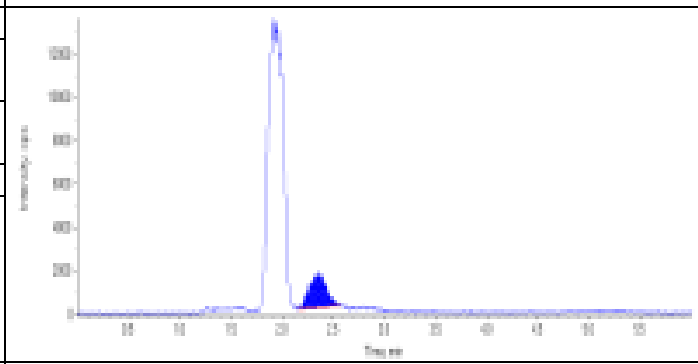
Recovery %	Amount found (ng/mL)	Amount added (ng/mL)	Conc. Of Vitamin A (ng/mL)	Replicate Samples
1	12.51	50	49.02	99.40
2	12.52	50	49.04	98.0
3	12.54	50	49.0	96.8
4	12.57	50	49.8	97.60

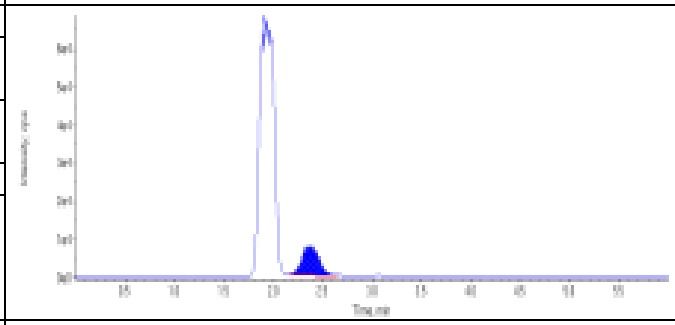
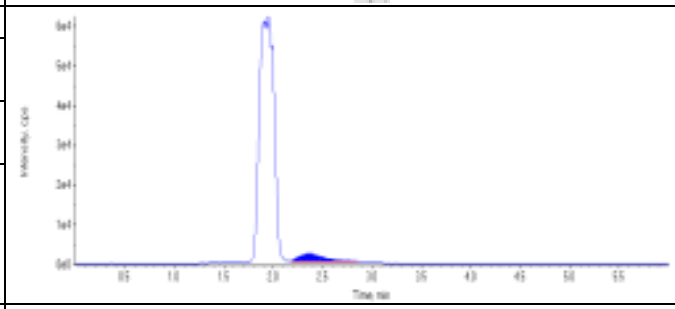
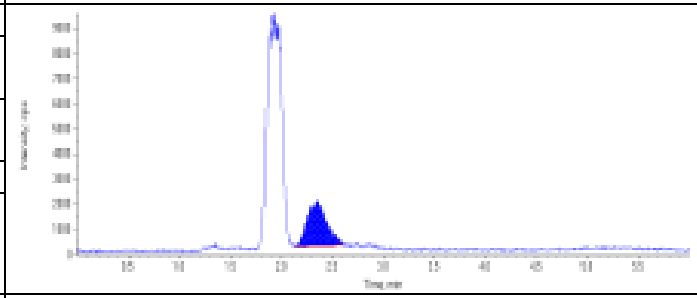
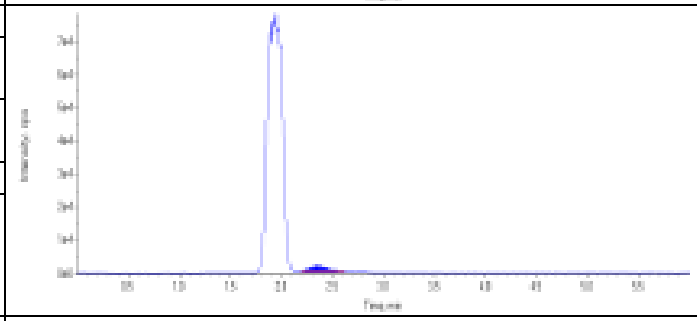
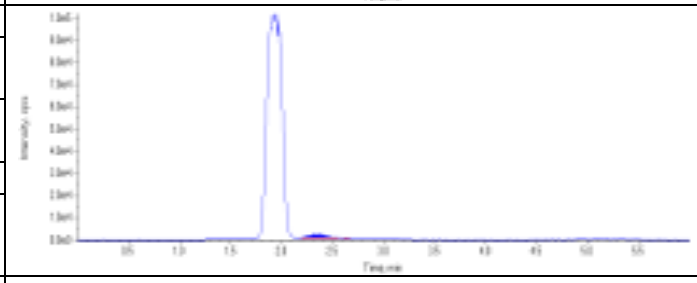
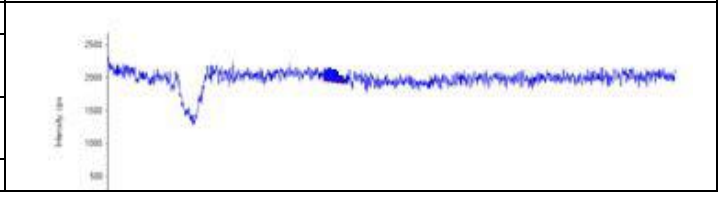
5	12.59	50	49.3	98.60
Average	12.546		49.032	98.080
SD	0.034		0.178	1.01
CV (%)	0.27		0.36	
LOD			0.53	
LOQ			1.78	
Error			-48.032	
95% CI			$49.03_2 \pm 0.22$	

Key: SD = Standard Deviation; CV = Coefficient of Variation; LOD = Limit of Detection; LOQ = Limit of Quantitation; CI = Confidence Interval.

Total ion chromatograms obtained from the LC/MS/MS is showed below. The peaks demonstrated sharp and symmetrical peak profiles.

STD1		
RT (Exp. RT):	***1.89 (1.89) min***	
Calculated Conc:	***44.0 ng/mL***	
Area:	***4.64e+004***	
Sample Type:	***(Standard)***	
STD2		
RT (Exp. RT):	***1.75 (1.76) min***	
Calculated Conc:	***20.8 ng/mL***	
Area:	***1.12e+004***	
Sample Type:	***(Standard)***	
STD3		
RT (Exp. RT):	***1.88 (1.89) min***	
Calculated Conc:	***210. ng/mL***	
Area:	***1.92e+005***	
Sample Type:	***(Standard)***	
STD4		
RT (Exp. RT):	***1.88 (1.89) min***	
Calculated Conc:	***267. ng/mL***	
Area:	***2.15e+005***	

Sample Type:	***(Standard)***	
STD5		
RT (Exp. RT):	***1.88 (1.89) min***	
Calculated Conc:	***396. ng/mL***	
Area:	***2.65e+005***	
Sample Type:	***(Standard)***	
STD6		
RT (Exp. RT):	***1.89 (1.89) min***	
Calculated Conc:	***499. ng/mL***	
Area:	***2.90e+005***	
Sample Type:	***(Standard)***	
STD7		
RT (Exp. RT):	***1.88 (1.89) min***	
Calculated Conc:	***764. ng/mL***	
Area:	***3.28e+005***	
Sample Type:	***(Standard)***	
STD8		
RT (Exp. RT):	***1.89 (1.89) min***	
Calculated Conc:	***922. ng/mL***	
Area:	***2.85e+005***	
Sample Type:	***(Standard)***	
SPL001		
RT (Exp. RT):	***2.36 (2.36) min***	
Calculated Conc:	***45.6 ng/mL***	
Area:	***1.81e+004***	
Sample Type:	***(Unknown)***	
SPL002		
RT (Exp. RT):	***2.35 (1.89) min***	
Calculated	***7.02 ng/mL***	

Conc:		
Area:	***3.19e+004***	
Sample Type:	***(Unknown)***	
SPL003		
RT (Exp. RT):	***2.39 (1.89) min***	
Calculated Conc:	***41.6 ng/mL***	
Area:	***9.32e+004***	
Sample Type:	***(Unknown)***	
SPL004		
RT (Exp. RT):	***2.34 (1.89) min***	
Calculated Conc:	***12.6 ng/mL***	
Area:	***3.70e+004***	
Sample Type:	(Unknown)	
SPL005		
RT (Exp. RT):	***2.34 (1.89) min***	
Calculated Conc:	***30 ng/mL***	
Area:	***2.22e+004***	
Sample Type:	***(Unknown)***	
SPL006		
RT (Exp. RT):	***2.38 (1.89) min***	
Calculated Conc:	***8.46 ng/mL***	
Area:	***2.33e+004***	
Sample Type:	***(Unknown)***	
SPL007		
RT (Exp. RT):	***2.31 (1.89) min***	
Calculated Conc:	***7.37 ng/mL***	
Area:	***2.60e+004***	
Sample Type:	***(Unknown)***	
De-Ionized		
RT (Exp. RT):	***1.89 (1. 89)*** min	
Calculated Conc:	***0.00*** ng/mL	
Area:	0.00	

Sample Type:	(Unknown)	

Figure 1: Total ion chromatograms of the pure vitamin A and the samples

Recovery and accuracy

The areas under the peaks obtained were used to calculate the absolute recovery from standard working solutions with the peak-areas from standard samples. The analyses of the reproducible samples were done and the results shown in Table 2 demonstrated that the average recovery of vitamin A (retinol) was 98.08%. The reproducibility of the analytical method was excellent and the coefficient of variation was 0.36%.

In order to detect this vitamin, and to validate the applicability and reliability of this technique with real samples, the proposed analytical technique has been applied to the analysis of seven Jordanian local fresh milk samples. The results were listed in Table 2. The results showed a remarkable presence of vitamin A in all the commercial local fresh milk samples analyzed.

Analytical applications

Table 2: Concentrations of Vitamin A in the pure Vitamin and commercial local fresh milk samples

Sample Name	**Sample Type**	**Area (cps)**	**RT (min)**	**Target [Conc]. (ng/mL)**	**Calculated Conc. (ng/mL)**
STD001	Standard	*4.640e+04*	*1.89*	*50.0*	*44.0*
STD002	*Standard*	*1.080e+05*	*1.76*	*50-100.*	*98.0*
STD003	*Standard*	*1.920e+05*	*1.88*	*100-250.*	*210*
STD004	*Standard*	*2.150e+05*	*1.88*	*250-300.*	*267*
STD005	*Standard*	*2.650e+05*	*1.88*	*300-400.*	*396*
STD006	*Standard*	*2.900e+05*	*1.89*	*400-500.*	*499*
STD007	*Standard*	*3.280e+05*	*1.88*	*500-750.*	*764*
STD008	*Standard*	*2.850e+05*	*1.89*	*750-1000.*	*922*
SPL001	*Unknown*	1.81e+004	*1.89*	N/A	45.6
SPL002	*Unknown*	3.19e+004	1.89	N/A	7.02
SPL003	*Unknown*	9.32e+004	*1.89*	N/A	41.6
SPL004	*Unknown*	3.70e+004	1.89	N/A	12.6
SPL005	*Unknown*	2.22e+004	*1.89*	N/A	30
SPL006	*Unknown*	2.33e+004	*1.89*	N/A	8.46
SPL007	Unknown	2.60e+004	*1.89*	N/A	7.37
De-IONIZE	Unknown	0.00	1.89	N/A	0.00

Key: RT = Retention Time; STD = Standard; SPL = Sample; N/A = Not Applicable.

The distributions of average concentrations of vitamin A in the samples are shown in the figure below.

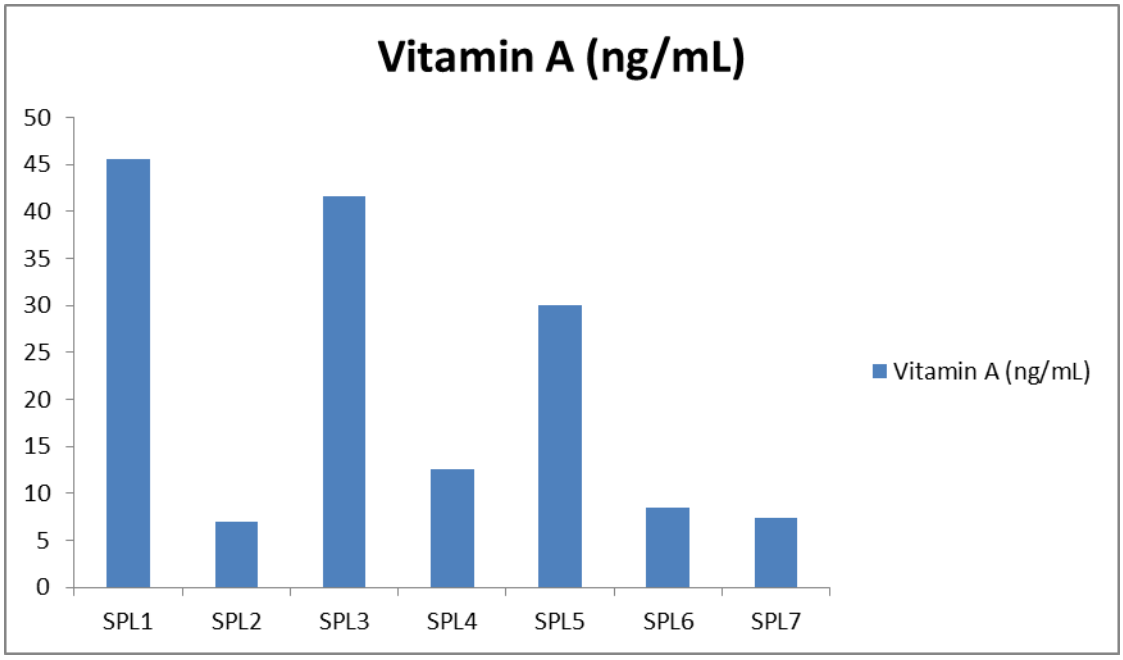


Figure 2. Graphical representations of the average calculated concentrations of vitamin A in the samples. The average concentrations are presented in ng/mL.

IV. Discussion

Seven Jordanian local fresh milk samples were analyzed for vitamin A. The type of vitamin A determined in this study was retinol. During standards and samples preparations, vitamin A acetate was used as an internal standard which resulted in systematic peak separation and resolution as demonstrated in figure 1.

The working standard ranges used were 0.0-1000.0 ng/mL. The total ion chromatogram revealed symmetrical peak profiles for both standards and samples as observed in figure 1. The standard yielded very high concentrations of vitamin A due to the fact that pure vitamin A was used thus resulted in high values of the average calculated concentrations of 20.8-922 ng/mL, retention time ranges of 1.76-1.89 min and area of the chromatograms ranges between 1.12e+004 and 3.28e+005 as listed in table 2. During the measurements, all the local fresh milk samples have remarkable concentrations of the vitamin A. The calculated concentrations of the local fresh milk samples were in the range 7.02-45.6 ng/mL as presented in figure 1 and table 2. These lower concentrations in comparison to that of the standards may be due to the samples fortification, preservation and more especially harsh storage conditions. The blank samples used have zero concentration of vitamin A as shown in figure 1 and table 2.

The retention times for all the samples was 1.89 min where as their area of chromatograms ranges between 1.81e+004 and 9.32e+004 as presented in Table 2. From figure 1 and table 2, sample 1 have the highest concentration of 45.6 ng/mL where as sample 2 have the lowest calculated concentration of 7.02 ng/mL. However, the average calculated concentrations of vitamin A in the local fresh milk samples analyzed were below the United States Council for Responsible Nutrition, 2011 and National Academy of Sciences, 1974 for the Reference Daily Intake of vitamin A of 400 µg/day for children and 900 µg/day for adults. Figure 2, also demonstrated clearly and graphically the distribution of individual sample concentration in the study.

In a related work in the simultaneous determination of vitamins A, E and β-carotene in bovine milk by high performance liquid chromatography–ion trap mass spectrometry (HPLC–MS^n), the vitamin A obtained at fragment ions m/z 213 and m/z 199 ranged between 36 µg/100mL and 59 µg/100mL [15] which is far less than the average calculated concentrations obtained in this work. In another for the determination of vitamin A and E in milk powder using supercritical fluid extraction for sample clean-up, they reported higher value of coefficient of variation of 4% and higher value of retention time of 15mins for vitamin A [3]. This implies that the present work is less susceptible to error and less time consuming owing to it lower value of CV of 0.36% and RT of 1.89 min. In compiling different extractions and chromatographic techniques for the determination of fat soluble vitamins, most of the methods used 2-50g of samples during the analyses [4], where as this work uses just 1g. Sodium hydroxide was used instead of the usual potassium hydroxide a factor which makes this work more unique compared to others. The technique used LC/MS/MS may be most promising due to its rapid chromatographic separation.

This present method was validated using repeatability studies, triplicate analyses, recovery experiments, proficiency study data and comparison with related literatures. The average recovery experiment obtained was reasonably found to be 98.08%, CV of 0.36%, SD of 0.178, LOD of 0.53, LOQ of 1.78 and error of -48.032 as showed in Table 1.

V. Conclusion

This work has reported the presence of remarkable concentration of vitamin A in all samples analyzed. The calculated concentrations of the Jordanian local fresh milk samples were in the range 7.02 - 45.6 ng/mL. However, the average calculated concentrations of vitamin A in the local fresh milk samples analyzed were below the United States Council for Responsible Nutrition, 2011 and National Academy of Sciences, 1974 for the Reference Daily Intake of vitamin A of 400 µg/day for children and 900 µg/day for adult. Further work should include solid phase extraction (SPE) instead of liquid liquid extraction (LLE) because SPE is more faster and its minimizes solvent lost.

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