

## COMPARATIVE STUDY BETWEEN PECTROPHOTOMETRIC AND HPLC METHODS USED FOR IODINE EXTRACTION IN MILK AND SOME DAIRY PRODUCTS

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### ABSTRACT

Determination of iodine in milk and its products is very important from the nutritional view point. Simple and rapid extraction methods were evaluated and validated for determining iodine in milk and some dairy products by Spectrophotometric method and HPLC methods. Iodine in milk (raw and pasteurized), white soft cheese and yoghurt were extracted by three methods first, with ammonium persulfate, second with acetic and nitric acid, third with alkaline ashing. After extraction, the reaction of  $As^{3+}-Ce^{4+}$  was performed at 32 °C, the transmission was measured spectrophotometrically at 420 nm. The results showed the precision of the ammonium persulfate method for all samples ( $CV < 4\%$ ) are higher than that of the alkaline ashing and acetic acid with nitric acid methods. The recoveries of iodine added to samples in range 91- 97 %, 93 – 120 % for extraction of milk iodine with ammonium persulfate, mixture of acetic acid with nitric acid, respectively, are higher compared with alkaline ashing. In addition, the linear coefficient ( $r$ ) for ammonium persulfate is 0.9994 better than mixture of acetic with nitric acid and alkaline ashing methods. The Comparison between spectrophotometric and HPLC methods for iodine analysis was carried on raw and pasteurized milk after extraction with the three extraction methods. The data showed reasonably strong correlation between results from both of those analytical methods ( $r = 0.9641, .9682, 0.9533$  for ammo. persulfate, acetic acid with nitric acid and alkaline ashing respectively). It was found that method HPLC gave comparable results to spectrophotometric method.

**Key words:** Iodine, Milk, Cheese, Yoghurt, Ammo. persulfate, acetic with nitric acid, ashing, Spectrophotometry, HPLC.

### INTRODUCTION

Milk is an important source of iodine (Pearce *et al.*, 2004). Iodine analysis is a valuable tool in assessing its intake of milk and it can be used as a routine method of quality control. Iodine is not only necessary for the production of thyroid hormones, but also it is responsible for the production of all the other hormones in the body.

Iodine concentration of milk has been determined by chemical methods (Dellavallae & Barbano, 1984; Holt *et al.*, 1989). Garwin (1994) compared with two colorimetric iodine assays that are based on different chemistry, one assay kinetically measured the initial iodine catalysis of the redox reaction between  $Ce^{+4}$  and  $As^{+3}$ , other monitored iodine catalysis of the reaction between thiocyanate and nitrite. The specific ion electrode has been successfully used to measure the iodine content of raw milk (Wallen *et al.*; 1982). Because of the interference of sulfhydryl that are produced when milk

is heat treated, the use of electrode method for determination of iodine in market milk is not recommended ( Bruhn & Franke , 1978).

Methods for the determination of iodine by HPLC are usually based on ion exchange chromatography. Iodine in milk is present almost exclusively in I<sup>-</sup> form ( Underwood, 1977) , and the quantitative of I<sup>-</sup> (vs. total I ) in milk using HPLC is a recognized official method (Association of official Analytical Chemists, 1993). Previously, Hurst *et al.*, (1983) described HPLC method for the extraction and analysis of iodine as iodide in milk, cocoa beans and milk chocolate. Moreover, Melichercik *et al.*,( 2006), comparison of HPLC with electrode method for determination of iodide in raw and processed milk. Other methods used to determine iodine in milk are inductively coupled plasma mass spectrometry (Vanhoe *et al.* 1993, Castro *et al.*, 2010) and neutron activation analysis ( James, 2001). Min-hao *et al.* (2006) determined micro amounts of iodine in milk powder by the derivable gas chromatography.

Many sample preparation techniques were used prior to iodine analysis as mineral distillation methods (Stole and Nemeth , 1961) and alkaline ashing methods (Stable – Taucher, 1975). Although numerous modifications have been made to both methods over the years, the precision and accuracy of these procedures were often hindered by interference of fat and proteins. Moreover the alkaline ashing method is time consuming , taking three days for one assay run. Whereas, the chloric acid method is most commonly used to obtain accurate and reproducible measurements of iodine and remove interfering substances (Benotti *et al.*,1965). Unfortunately chloric acid is a potential hazard, many laboratory ,especially in developing countries, do not have the appropriate equipment or resources to purchase the special perchloric acid fume hood required by standard laboratory safety regulations . Therefore, researches for inexpensive reliable methods and instrumentation are needed. Generally, the review of iodine determination on a wide-ranging set of complex matrices is not available. The aim of the present study is conducted to evaluate and validate simple , rapid and economic methods to determine the iodine content in milk and some dairy products samples based on spectrophotometric method to compare with HPLC results .

## **MATERIALS AND METHODS**

Whole raw buffalo milk was obtained from Mahalate Moussa Station, Animal Production Research Institute, (ARC). Pasteurized buffalo milk was heated at 75 °C for 15 sec. and then cooled to 4 °C. UF White soft cheese (3% salt) and yogurt were obtained from Dairy Processing, Animal Production Research Institute. Potassium iodate used as standard was purchased from Sigma Chemical Co. Ammonium persulfate (ammonium peroxydisulfate, (NH<sub>4</sub>)<sub>2</sub>S<sub>2</sub>O<sub>8</sub> ) , arsenic trioxide, concentrated sulfuric acid ( 98% ) were obtained from Prolabo Co. Deionized water was used for preparation of reagents and dilution procedure: Iodine was extracted from all milk samples before analysis by three procedures:

**1- Extraction with ammonium persulfate according to Pino *et al.*, (1996):**

One ml of milk sample was mixed with 5 ml of ammonium persulfate (1mol /L). The mixture was heated for 30 min at 95°C in water bath, cooled, and the solution was filtered, the filtrate was undertaken for iodine determination .

**2- Extraction by acetic acid and nitric acid according to Dionex Corporation (1996) :**

50 ml of milk was transferred into 100 ml volumetric flask and 4.0 ml of 3 % acetic acid was added , then the solution was mixed for 10 sec. by vortexing . This was followed by addition of 1 ml concentrated nitric acid and well mixed. The content of volumetric flask was diluted to 100 ml with deionized water and mixed thoroughly by vortexing . The mixture was then centrifuged at 4000 rpm for 15 min. and filtrated. 1ml of the filtrate was diluted by adding 3 ml of deionized water.

**3 –Extraction by alkaline dry ashing according to Garwin *et al.*,(1994):**

About 3 hours, then heated at 145 °C for 19 hours (until all black fog is completely released), then, transferred into muffle furnace for ashing at 550 °C for 4 hours. The ash dissolved in 6 ml H<sub>2</sub>O and filtrated. 1.2 ml of the filtrate was used to iodine determination by As<sup>3+</sup> - Ce<sup>4+</sup> catalytic One ml of milk plus 1 ml KOH (11.08 M) were heated in an oven at 105°C for spectrophotometric method. In case of cheese or yoghurt 0.5 gm was taken for extraction

Total iodine concentrations in the extraction of raw and pasteurized milk , cheese and yoghurt samples were measured by As<sup>3+</sup>- Ce<sup>4+</sup> catalytic spectrophotometric method. Extracted raw and pasteurized milk were subjected to HPLC analysis .

**1- Catalytic spectrophotometric method:** Sulfuric acid (2.5 mol/L) was prepared in ice bath by carefully adding 280 ml of concentrated sulfuric acid to 1000 ml of deionized water. The cold mixture was diluted to 2000 ml. Ceric ammonium sulfate was prepared by dissolving 1 g of ceric ammonium sulfate in 100 ml 1.25mol /L.

Arsenious acid (0.0253 mol / L) was prepared by dissolving 1 g of arsenic trioxide, and 5g of sod. chloride in 40 ml 2.5 mol / L sulfuric acid and heating on hot plat until dissolved. The cold mixture was diluted to 250 ml with deionized water.

1.2 ml of each extracted samples was transferred into test tube and 2.0 ml of arsenious acid, 1ml of 1.25 mol/ L H<sub>2</sub>SO<sub>4</sub>, and 1ml of deionized H<sub>2</sub>O were added. The tubes were then placed in a 32 °C and incubated for 10 min. The reaction is started by adding 0.5 ml of ceric ammonium sulfate to all tubes, which were incubated for 10 min. At the end of the incubation, the percent transmission was read at 420 nm. Deionized water was used to adjust the spectrophotometer to 100 % transmission. In zero concentration sample was replaced with deionized water and treated as described above.

**2-HPLC method: A method for the determination of iodine by reversed-phase high-performance liquid chromatography has been carried out.**

**The filtrate was subjected to separation by HPLC with the following conditions:**

The mobile phase at flow rate 1 ml/min; was acetonitrile and methanol 70 : 30 ( v/v). Agilent 1100 series (Waldborn, Germany), quaternary pump (G1311A), Degasser (G1322A), thermostated autosamples (G1329A), variable wave length detector (G1314A); and column: Zorbax 300SB C<sub>18</sub> (4.5 X 250 mm) ( Agilent Technologies, USA). Injection was carried out at wave length 240 nm for separation. Recovery was carried out for the two methods by adding known concentrations of standard iodine ( potassium iodate) to tested samples and calculated by the equation (A O A C, 2002):

$$R \% = ( C_s - C_p / C_a ) . 100$$

Where R (%) is the percent recovery of added standard; C<sub>s</sub> is iodine concentration in the spiked sample ; C<sub>p</sub> is iodine concentration in the original sample and C<sub>a</sub> is iodine concentration of standard iodine. Precision for the two methods was measured within a laboratory as coefficient variation (CV %) Coefficient variation, which was calculated as the equation ( Horwitz, 2003) :

$$CV (%) = ( SD / mean ) . 100$$

## **RESULTS AND DISCUSSION**

### **The catalytic spectrophotometric method:**

Determination of iodine content in milk and some dairy products need for an efficient and reliable extraction and quantification of total iodine .The As<sup>3+</sup> - Ce<sup>4+</sup> catalytic spectrophotometric method is based on measuring transmittance at 420 nm due to decrease with time . Fig (1) showed the linearity of catalytic spectrophotometric method with three extraction methods, ammo. persulfate, acetic acid with nitric acid and alkaline ashing . The concentration of iodine in milk and dairy products samples were calculated according to standard curves and the equation (1), (2) and (3) for extraction of ammo. persulfate, acetic acid with nitric acid and alkaline ashing respectively.

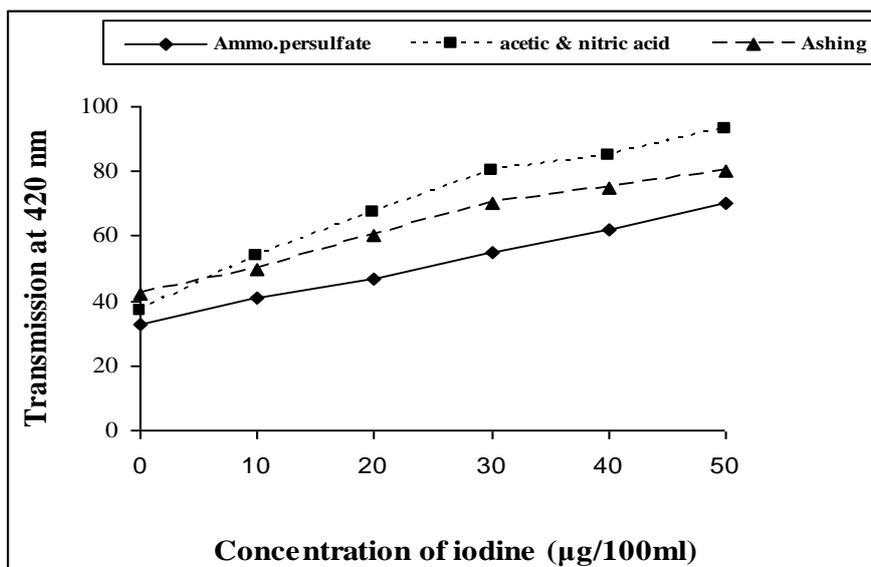
$$Y = 33.047 + 0.731 X \quad (1)$$

$$Y = 41.761 + 1.103 X \quad (2)$$

$$Y = 43.190 + 0.786 X \quad (3)$$

Y and X represent the transmittance at 420 nm and concentration of iodine (µg/100 ml) respectively.

The results showed that the linearity was affected by the extraction methods . Comparing standard curves , the ammo. persulfate had a steeper slope and the linear range (0 – 50 µg / 100 ml), while , the linear range of both acetic acid with nitric acid and alkaline ashing methods was 0 - 30 µg / 100 ml. The correlation coefficients (r), for ammo. persulfate was higher (0.9994), compared with acetic acid & nitric acid (0.9824) and alkaline ashing (0.9906) methods. The results of linear analyses indicated that catalytic spectrophotometric method with three extraction methods was reliable for quantifying iodine, while ammo. persulfate method was very reliable.



**Fig (1): The standard curves of iodine concentration in extractions using spectrophotometric method for determination**

The efficiency and validation of these methods were evaluated by determination the recovery of iodine added to milk, cheese and yoghurt samples at two concentrations (5 & 25 µg / 100 ml) as shown in table (1). The range of mean recovery of iodine added to all samples treated with ammo. persulfate was 87 – 95 % with the coefficient of variability (CV%) smaller than 4 % ranging from 2.1 to 3.5 %. The average recovery in milk samples was higher than those for cheese samples. It was found that the results obtained by ammo. persulfate method were higher (2 – 8 %) compared with traditional ashing method. For the acetic acid & nitric acid extraction the recoveries of the added standard iodine were 91 – 121 % with the coefficient of variability (CV%) smaller than 8 %..While the iodine recovery of milk samples treated with acetic & nitric acid reached 119- 121 with CV % up to 7.5%,. this might be due to treating milk sample with nitric acid which caused instability and interfering with  $As^{3+}$  -  $Ce^{4+}$  reaction which monitoring by spectrophotometer .Such an excessive recovery did not occur when cheese and yoghurt samples were treated with acetic acid & nitric acid. These results are in agreement with Vanhoe *et al.* (1993) who showed that the oxidation state had a strong impact on the analytical performance and found excessive recovery of iodine in milk powder treated with nitric acid. Hence, determination of iodine in milk samples, treated with acetic & nitric acid is less suitable for spectrophotometric method and the recovery should be carried out every assay run to correct the results. While, ammo. persulfate method was more suitable for spectrophotometric method and acceptable recovery on all samples was obtained .

These results emphasize the need of recovery routinely, in order to be aware of probable losses or excessive during sample preparation

.However , no correction for the recovery was included in the iodine values presented in table ( 2 ).

**Table (1): Recoveries of iodine added to milk cheese and yoghurt samples with different extractions using spectrophotometric method**

Samples	Iodine added to samples (µg/100ml)	Ammo. Per sulfate			Alkaline ashing			Acetic with nitric acids		
		Mean	± SD	(%) CV	Mean	± SD	(%) CV	Mean	± SD	(%) CV
Milk	5	91	3	3.3	84	5	6.0	119	8	6.7
	25	95	2	2.1	87	6	6.9	121	9	7.4
Cheese	5	87	3	3.5	85	5	5.9	94	6	6.4
	25	89	3	3.4	87	4	4.6	93	7	7.5
Yoghurt	5	92	2	2.2	86	4	4.7	93	5	5.4
	25	94	3	3.2	89	5	5.6	91	4	4.4

The results of catalytic spectrophotometric method with ammonium persulfate , acetic with nitric acid and alkaline ashing extraction methods are given in table (2) and Fig (2). Iodine level of raw milk, pasteurized milk, cheese and yoghurt samples with ammonium per sulfate were 5 - 15 % higher compared to those with alkaline ashing respectively. While, the results showed that iodine content in all samples were higher 13 -26 % when extracted by acetic with nitric acid compared to those samples with alkaline ashing . The results of alkaline ashing showed depresses the catalytic effect of iodine in the  $As^{3+} - Ce^{4+}$  reaction, this is in agreement with result of Belling (1983). Overall, the major problem associated with the alkaline method for iodine determination in milk samples is time consuming in the assay.

Fiedlerova, (1998) reported that spectrophotometric method is suitable for determination of total iodine in foods. It is based on  $As^{3+} - Ce^{4+}$  reaction carried out after preliminary alkaline dry ashing in the presence of KOH,  $ZnSO_4$  and  $KClO_3$ .

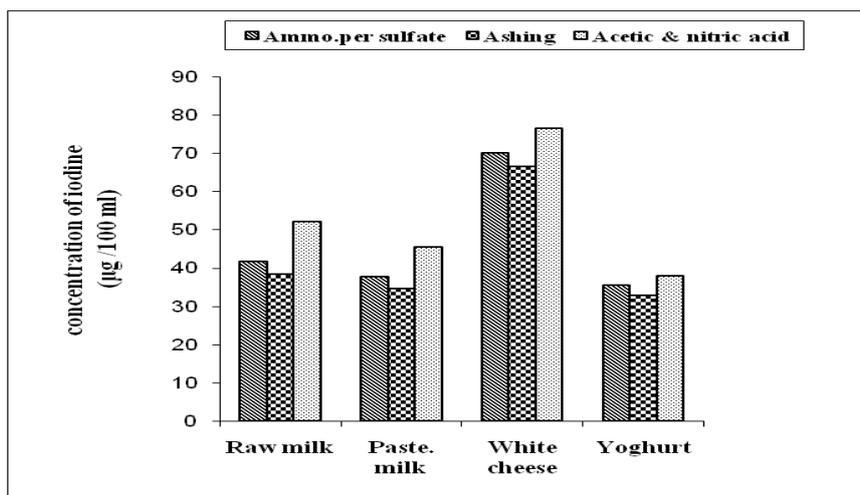
The precision of these methods were measured within a laboratory as coefficient variation (CV%) as shown in table (2 ).The CVs% of ammo. Persulfate method to determine iodine in raw milk, paste. milk, cheese and yoghurt were smaller than 4 % , demonstrating good precision. The data showed the precision of ammo. persulfate was higher compared with the traditional of alkaline ashing(  $CV < 6$  ) . Previous study of Amount *et al.*, (1986) found precision of ashing with spectrophotometric method  $< 8\%$  for milk and urine. While, the precision of acetic with nitric acid was lower than alkaline ashing. Although acetic acid with nitric acid gave higher iodine values for milk , cheese and yoghurt ,it was less precision compared with ammo. persulfate. Also, it was noticed that the precision of acetic acid with nitric acid was higher with cheese and yoghurt than milk. This result showed that acetic acid with nitric acid is more suitable for determination of iodine in yoghurt and cheese than milk.

As ammo. per sulfate extraction was not applied and investigated for iodine determination in milk and its products previously . While, Pino *et al.*, (1996) used ammonium per sulfate as a safe alternative oxidizing reagent for determination of urinary iodine. Moreover, Ding-you *et al.*( 2006) verified

that method for determination of urinary iodine. Their results showed that the ammo. per sulfate method, well correlated to the standard linear curve, precision and accurate. These results were agreement with present finding.

**Table (2): Determination of iodine in milk, cheese and yoghurt samples with different extractions by spectrophotometric method**

Samples	Concentration of iodine ( $\mu\text{g}/100\text{ ml}$ )								
	Ammo. Per sulfate			Alkaline ashing			Acetic with nitric acids		
	Mean	$\pm$ SD	(%) CV	Mean	$\pm$ SD	(%) CV	Mean	$\pm$ SD	(%) CV
Raw milk	41.65	1.41	3.4	35.56	2.10	5.9	52.11	3.60	6.9
Past. milk	37.16	1.35	3.6	31.23	1.73	5.5	45.51	2.87	6.3
White soft cheese	70.12	2.70	3.9	66.57	3.47	5.2	76.45	4.48	5.9
Yoghurt	35.54	1.10	3.1	32.84	1.51	4.6	37.86	1.85	4.9



**Fig. (2): Catalytic spectrophotometric results of iodine ( $\mu\text{g}/100\text{ ml}$ ) in raw & paste. milk, white soft cheese and yoghurt with different extractions**

**HPLC method**

Standard curve of potassium iodide was represented in Fig.(3). The correlation between area count and concentration of potassium iodide showed an excellent linear response with  $r = 0.9997$ . HPLC separated iodine in milk as iodide as reported by Hurst *et al.*, (1983) who described HPLC method for the extraction and analysis of iodine as iodide in milk, cocoa beans and milk chocolate. Also ,previous study of Underwood ,1977) showed that iodine in milk exist primarily in the ionic iodide forms. Iodine concentration was calculated by multiple concentration of potassium iodide by 76.5 % ( Molecular weight KI = 166 g , K = 39 , I = 127 thus, percentage of iodine in KI = 76.5 %).

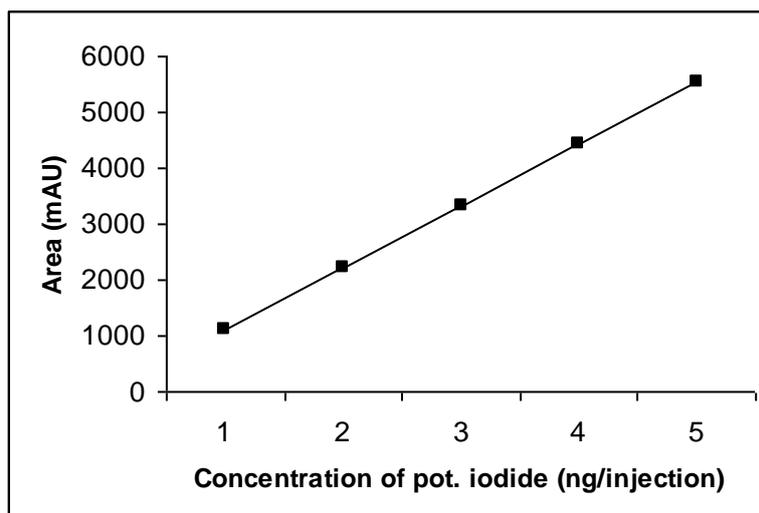


Fig.(3): Standard curve of potassium iodide for determination of iodine in milk by HPLC

Table (3) shows the mean recoveries of HPLC method were 98 % , 104 % and 94 for ammo. Persulfate , acetic acid & nitric and alkaline methods respectively . The high recovery demonstrated good efficiency of HPLC method . The results obtained coincide with Melicherik *et al.*, (2006), they found recovery of iodine added to milk using ion HPLC method between 91 – 100 %.

Table (3): Recoveries of iodine added to milk samples with different extractions using HPLC

Samples	Iodine added to samples (µg/100ml)	Ammo. Per sulfate			Alkaline ashing			Acetic with nitric acids		
		Mean ± SD	(%)	CV	Mean CV	± SD	(%)	Mean ± SD	(%)	CV
Raw milk	25	98	3	3.1	94	4	4.3	104	4	3.8

The HPLC results for three extraction methods are given in table (4). The results indicated that iodine levels in raw and heated milk were similar for both ammo per- sulfate and acetic and nitric acid extraction methods. While those were higher compared to alkaline ashing method. The precision (CV %) of ammo. persulfate and acetic acid & nitric acid method (less than 3) were slightly higher compared to alkaline ashing (less than 4).

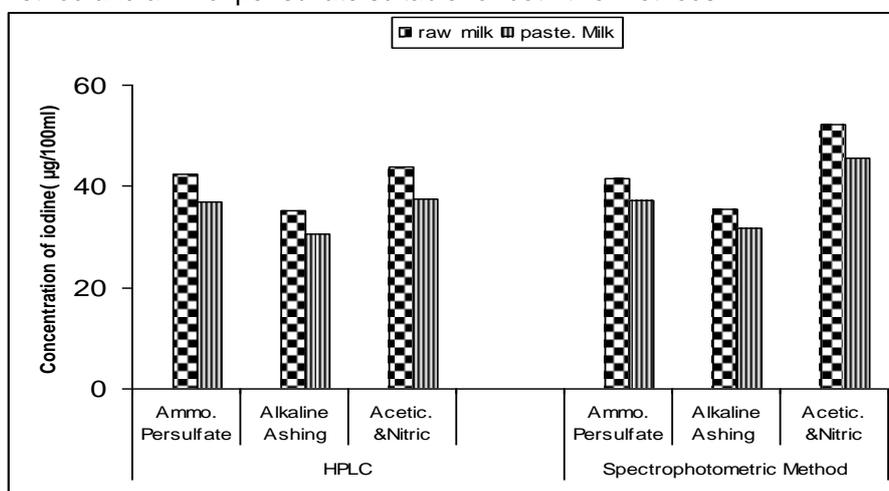
Table (4): Determination of iodine in milk samples by HPLC method with different extractions

Samples	Concentration of iodine (µg/100 ml)
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	Ammono. Per sulfate			Alkaline ashing			Acetic with nitric acids		
	Mean	± SD	(%) CV	Mean	± SD	(%) CV	Mean	± SD	(%) CV
Raw milk	42.32	0.89	2.1	35.33	1.30	3.7	43.82	1.19	2.7
Paste. Milk	36.86	0.96	2.6	30.50	1.08	3.5	37.62	1.10	2.9

**Comparison between catalytic spectrophotometry and HPLC methods**

A comparison between two methods, spectrophotometric and HPLC methods for the determination of iodine in raw and pasteurized milk is presented in Fig. (4). The results obtained of iodine determination in raw and heated milk samples by C<sub>18</sub> reverse phase HPLC UV detection, after preliminary ammonium persulfate, and alkaline ashing, agreed with As<sup>3+</sup> - Ce<sup>4+</sup>-catalytic spectrophotometric method. In contrast, the result of HPLC with acetic and nitric acid was 21% lower than that obtained from As<sup>3+</sup> - Ce<sup>4+</sup>-catalytic spectrophotometric method. This result emphasized that mixture of acetic and nitric acid extraction interferes with As<sup>3+</sup> - Ce<sup>4+</sup>-catalytic spectrophotometric method. However, the results indicated that iodine levels in raw and pasteurized milk were similar for both ammonium persulfate and acetic and nitric acid. This indicated that acetic acid and nitric extraction method were more suitable for HPLC than catalytic spectrophotometric method and ammonium persulfate suitable for both two methods.



**Fig. (4): Comparison of mean iodine content per raw and heated milk measured by spectrophotometric and HPLC methods**

The results showed that the HPLC and catalytic spectrophotometric methods were more comparable in the recovery with ammonium persulfate and alkaline ashing than those with acetic and nitric acid as shown in Fig (5).

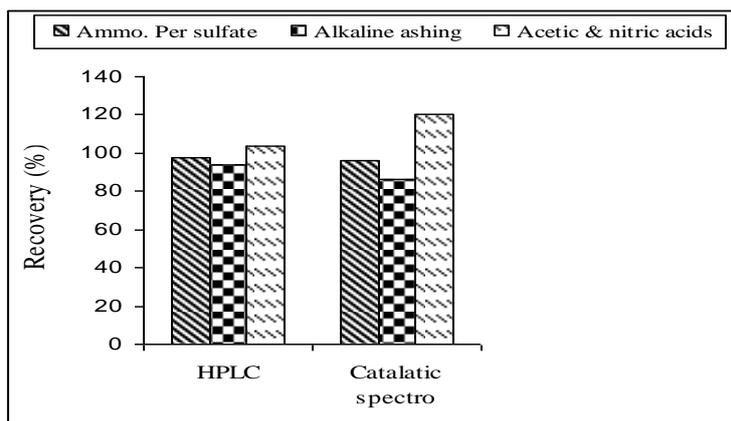


Fig. (5): Comparison of mean recovery of iodine added to milk measured by spectrophotometric and HPLC methods

Comparison of total milk iodine measured by the spectrophotometric with milk iodine determined by HPLC showed a high correlation  $r = 0.9641$ ,  $y = 1.305 + 0.921x$ ;  $r = 0.9682$ ,  $y = -5.355 + 1.333x$  and  $r = 0.9533$ ,  $y = 1.546 + 0.983x$  for ammo. per sulfate, acetic acid with nitric acid and alkaline ashing respectively

Y is the value of iodine by spectrophotometric method and x is the value of HPLC method

## CONCLUSION

Ammo. persulfate as oxidizing agent inexpensive is rapid, precision and efficiency method for determination of iodine in milk and dairy products based on spectrophotometric and HPLC methods. While, low cost, and simple spectrophotometer instrument make this method more suitable to assessment of iodine determination in developing countries

As, acetic acid with nitric acid extraction method is rapid and low cost for determination of iodine by spectrophotometric method, it is less precision and stable compared with ammo. persulfate. But, this method is high precision and efficiency for determination of iodine by HPLC.

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### دراسة مقارنة بين طرق استخلاص اليود المختلفة لتقديره في اللبن وبعض المنتجات اللبنية بالطريقة الطيفية وطريقة HPLC ماجدة عبد العزيز و كريمة أبو العينين قسم بحوث كيمياء الألبان- معهد بحوث الإنتاج الحيواني

تقدير محتوى اليود في اللبن ومنتجاته من الأمور الهامة في مجال التغذية. ويحتاج لطرق دقيقة ذات كفاءة عالية لاستخلاصه أولاً ثم تقديره كميًا. ونظرًا لارتفاع تكاليف تقدير اليود بما لا يلائم سب البلدان النامية لذلك هناك ضرورة للبحث عن طرق دقيقة و غير مكلفة ويمكن الاعتماد عليها. ولهذا فإن الدراسة الحالية هدفها الرئيسي إيجاد طرق إستخلاص بسيطة وسريعة واقتصادية لتقدير اليود في اللبن وبعض منتجاته معتمدة على الطريقة الطيفية و مقارنتها بطريقة HPLC وقد تم استخلاص اليود من اللبن (الخام والمبستر)، والجبن الأبيض الطرى واللبن الزبادى بثلاث طرق الأولى، مع بيرسلفات الأمونيوم ، والثانية مع حمض الخليك وحمض النيتريك، والثالثة مع الحرق . بعد الاستخلاص، تم إجراء التفاعل  $Ce^{4+} - AS^{+3}$  عند ٣٢ درجة مئوية و قياس الإنبعاث الطيفي عند ٤٢٠ نانومتر وقد أظهرت النتائج أن :

طريقة بيرسلفات الأمونيوم ، لجميع العينات ( $CV > 4\%$ ) أكثر دقة من النتائج المتحصل عليها بطرق الحرق وحمض الخليك مع حامض النيتريك . وقد أجريت مقارنة بين الطريقة الطيفية و HPLC لتحليل اليود في اللبن واللبن المبستر بعد الاستخلاص مع الطرق الثلاثة السابقة . فقد وجد أن طريقة طيفية أعطت نتائج مماثلة لطريقة HPLC. وأظهرت النتائج أن:

درجة العلاقة بواسطة معامل الارتباط بين الطريقة اللونية و طريقة HPLC لقياس اليود هي ٠,٩٦٤١، ٠,٩٦٨٢، و ٠,٩٥٣٣ لنتائج بيرسلفات الأمونيوم ، وحمض الخليك مع حامض النيتريك و الحرق على التوالي. و كما أظهرت النتائج أن الطريقة الطيفية أعطت نتائج دقيقة مماثلة لطريقة HPLC وتستخلص من هذه الدراسة أن استخلاص اليود بواسطة طريقة بيرسلفات الأمونيوم وتقديره بالطريقة الطيفية مناسبة تمامًا لتقدير اليود في اللبن ومنتجاته من حيث الدقة والسرعة وإنخفاض التكلفة.

#### قام بتحكيم البحث

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