Food Science and Quality Management
ISSN 2224-6088 (Paper) ISSN 2225-0557 (Online) DOI: 10.7176/FSQM
Vol.83, 2019

Spectrophotometric Determination of Oxalic Acid in Dietary Sources Through Catalytic Titration with Hexavalent Chromium

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Abstract
Excess oxalic acid is thought to be a key factor of kidney stone formation in humans. Thus its level in food stuffs should be monitored. In this study, a new spectrophotometric titration method was developed for the determination of oxalic acid (OA) in dietary sources through reaction with hexavalent chromium in presence of Mn (II) as a catalyst. Cr (VI) was used as UV-active material and the determination of OA was based on the decrease in absorbance of the Cr (VI) solution as a result of its reaction with OA. Factors influencing the reduction of Cr (VI), including incubation time, solution pH and background concentration ratio have been optimized. The results showed that the amount of oxalic acid (mg/g) in vegetable samples of Ethiopian collared green (raw and cooked), cabbage (raw and cooked), lettuce (raw),beetroot (raw), pineapple (before ripe and fully ripe) and mango (before ripe and fully ripe) were 24.15 ± 0.565 and 12.05 ± 0.183, 16.72 ± 0.388 and 9.50 ± 0.424, 14.44 ± 0.183, 15.77 ± 0.175, 23.08 ± 0.212 and 18.31 ± 0.424, 6.75 ± 0.212 and 1.18 ± 0.169 respectively. Ethiopian collared green *(Brassica oleraceavar. acephala)* contained highest concentration of oxalate, but it was reduced by 50 % after boiling for ten minute. The level of oxalate content in the analyzed fruit samples was found to decrease with increasing growing stage or state of ripeness. All studied food samples contained relatively moderate amounts of total oxalate (< 25 mg/g dry weight) and are safe for human consumption especially when cooked or fully ripe. A very good correlation was observed when the results from the current method and standard method (HPLC) were compared. In conclusion, the current method can successfully be used for determination of OA in fruit and vegetable samples.
Keywords: Spectrophotometric titration, Hexavalent Chromium, Oxalic acid, HPLC.
DOI: 10.7176/FSQM/83-04

Introduction

Oxalic acid is a product of protein metabolism and is one of the important nutrients in the human diet. Common dietary sources of oxalic acid include spinach (Spinacia oleracea), beet leaves, vegetables, chocolate, tea (Camellia sinensis) etc. [1]. Soluble oxalic acid can be found as potassium, sodium, or ammonium oxalate salts. Its levels depend on the type and age of plant tissue as well as growth rate [2-4].

Oxalic acid is of scientific interest as a result of its antinutritive properties and association with kidney stone formation at high concentrations. Oxalic acid (or its dissociated form oxalate) cause decreased bioavailability of other nutrients and increased risk of kidney stone formation due to production of insoluble salts with dietary cations (mainly calcium) thus rendering the complex unavailable for adsorption and assimilation (antinutritive effect). Adsorbed oxalic acid can also cause assimilated calcium to be precipitated as insoluble salts accumulating in the renal glomeruli, leading to renal disorder. Evidences showed that about 75% of all kidney stones are composed primarily of calcium oxalate and hyperoxaluria is a primary risk factor for this disorder [5-10]. Determination of oxalate in clinical samples such as urine and blood has thus served as a basis in diagnosis and medical management of primary and secondary hyperoxaluria, urinary stone disease [7, 11].

Reductions of oxalates in the diet can be achieved by avoiding foods which are known to contain high levels of oxalate and by processing the foods in different ways. Soaking and boiling are efficient way of reducing the level of oxalate in food if the cooking water is discarded [11]. Likewise, addition of calcium can make soluble oxalate unavailable for absorption by binding- complex [12]. By consuming a food high in calcium together with foods containing oxalates, the insoluble calcium oxalate formed passes through the intestinal tract without absorption and thereby decreases the risk of kidney stone formation. Other studies have shown that fat and oils can bind oxalate and thereby make soluble oxalates unavailable for absorption [13].

The oxalate determination by AOAC (1984) [14] method was based on calcium oxalate precipitation. It has some limitation in terms of sensitivity, precision and time consumption. Spectrometric methods have received considerable attention because of the significant advantages in the determination of several analyte at trace levels, especially when only a simple photometer is required as the main instrument. Spectrophotometric methods based on the inhabitation or the catalytic effect of OA on the rate of a definite reaction system has been reported [5, 7, 11, 12].

This paper describes a simple and sensitive method for determination of oxalic acid in selected green leafy vegetables (beetroot, cabbage, Ethiopian collared green and lettuce) and stony fruits (mango and pineapple) based on spectrophotometric titration of Cr (VI) by the analyte in the presence of Mn (II) as a catalyst. The

decrease in absorbance of the Cr (VI) was measured at 350 nm under optimized conditions. Method validation was carried out through determination of % recovery and comparison with standard technique-high-performance liquid chromatography (HPLC) [15-18].

Materials and Methods

Chemicals and Reagents

All the chemicals used were of analytical grade reagent and used without further purification.98% H_2SO_4 (analytical reagent, England), 37 % HCl (Aldrich, Germany), LC–MS-grade octanol (Sigma, Milwaukee, USA),50mM KH_2PO_4 as mobile phase and CH_3CN as organic solvent modifier were used. $MnCl_2$, as source of divalent manganese (used as catalyst) and $K_2Cr_2O_7$as source of hexavalent chromium (used as oxidant) (analytical reagent, Germany) was used during reduction of hexavalent chromium by oxalic acid. H_3PO_4 was used to adjust the initial pH of a solution to desired values during UV-Vis analysis. Distilled water was used for sample preparation, dilution and rinsing apparatus before sample solution preparation.

Apparatus and Instrument

UV-Vis spectrophotometer equipped with deuterium lamp, Quartz cuvette with 1 cm cell of path length (model CECIL 121, England)], orbital shaker, shaking bottle, glassware's, 0.045γm syringe filter, A refrigerator (Hitachi, Germany), digital analytical balance (ADAM, Model AFP-110L, England). Drying oven (Digit heat, J. P. Selecta, Germany), Digital pH, measuring cylinders (Duran, Germany), dropper, pipettes and micro pipettes (Pyrex, England), Volumetric flasks with stopper (Pyrex, England), Pyrex beaker to boil the real sample, aluminum foil, Whatman 541 filter paper, knife, spatula, wash bottle, hot plate**,** Pestle and mortar were used for grinding and homogenizing the fruit and vegetables samples. An Agilent technology (1200 infinity series) HPLC equipped with UV detector was used for determination of oxalate content.

Sample collection

Samples were collected systematically from common market and fruit juice shops of Hawassa town. The fully ripe and unripe stony fruits of pineapple and mango were purchased from fruit juice shops in Hawassa, whereas the green leafy vegetable of lettuce, beetroot and cabbage and Ethiopian collared green were collected from Hawassa common market.

Sample preparation

The vegetables were washed with tap water and edible parts of all the vegetables were selected. The raw portion of these samples were boiled for ten minute at room temperature and then cooled in cold water; the water was discarded. All of the vegetables except beetroot were vacuum dried for 24 hour at 50 °C and then vacuum packed until analysis. The vacuum dried materials were then ground into a fine powder by using mortar and pestle. After the edible part of the raw lettuce and beetroot was selected, it was chopped in to pieces then it was vacuum dried at 55 °C for two days. The vacuum dried materials were then ground into a fine powder by using mortar and pestle. M**ango and pineapple** were washed with tap water and the flesh part of the fruits was selected using knife then sliced in to smaller pieces and dried in an oven at 55 °C for 5 days. After the dried samples were ground into powdery form using mortar and pestle. The powdered samples were sieved to obtain fine powder which was later digested. Finally the powdered materials were sealed in an aluminum foil bag until analysis could commence.

Sample extraction for OA determination using UV-Vis Spectrophotometer method

1 g dried sample was weighed and boil in 150 ml water containing 27.5 ml 6 M HCl plus 2 drops of octanol for 25 min in beaker. The mixture was cooled, transferred to a 250 ml volumetric flask and made up to mark. The mixture was then filtered through Whatman 541 filter paper. ***A volume of 10 ml of this filtrate was evaporated at 40-45***^{o}C in a vacuum oven and re-dissolved in 10 ml of 0.01 M H_2SO_4. Then 0.5 mM $K_2Cr_2O_7$ and 0.25 mM $MnCl_2$ was prepared in 250 ml volumetric flask; this solution was the background (blank) solution during OA standard solution. 1 ml from each extracted sample and 9 ml from the blank solution, a total of 10 ml was taken for each sample and incubated for 60 min. Finally the total OA in the sample was analyzed using a UV–Vis Spectrophotometer [19].

Sample Extraction for OA determination using HPLC method

4 g of homogenized sample was weighed in to a 250 ml shaking bottle and 50 ml of 2N HCl was added. The content of the bottle was shaking for 50 min at 250 rpm then it was removed from the shaker and 50 ml HPLC grade H_2O was added, then appropriate volume of extract was filtered through 0.45µm syringe filter. The filtrate was transferred in to a 2 ml vial and the vial was capped. Finally, the total oxalate in the sample was analyzed using HPLC method.

Data analysis

As the study aimed to determine OA in dietary sources using UV-Vis spectrophotometer and HPLC method data obtained were analyzed by a computer program to analyze tabulated data using Microsoft Excel 2007 and origin 8 Software. T-test was used to assess the significance difference between fully ripe and unripe fruits and also raw and cooked vegetables.

Results and Discussion

Method development and optimization

In this study the amount of total oxalic acid in selected dietary sources (leafy vegetables and stony fruits) was determined using indirect UV-Vis method through Catalytic Titration with Cr (VI). Cr (VI) was used as UV-active material and the determination of OA was based on the decrease in absorbance of the Cr (VI) solution as a result of its reaction with OA in presence of Mn (II) as a catalyst. When the Cr (VI) was reacted with oxalic acid in the presence of Mn (II), it was reduced and gradually eliminated from the reaction. The advantage of this method is that it does not necessarily require completion of the reaction. Because, the decrease in absorbance of the solution after a specified duration of incubation depends on the residual concentration of oxidant which is used as limiting reactant (analyte). The kinetics of the reaction depends on the concentrations of all reactant and catalyst as well as other experimental variable including pH, and Temperature. The reaction of Cr (VI) with OA was catalyzed by Mn (II), but the amount of Mn (II) required to complete the reaction depends on how much Cr (VI) is present in the sample and thus affects the rate of reaction. Therefore, for successful application of the current method, it was important to optimize the experimental variables including oxidant to catalyst ratio, pH, and contact time. Prior to anything, however, it was important to determine whether a significant change in absorbance of the oxidant solution can be observed at high enough concentration of the analyte during incubation the two for different period of time. This was successful and the result is indicated in Figure 1 (A). In absence of the analyte (OA), the absorbance of the oxidant solution containing the catalysts remained practically unchanged. However, a progressive decrease in absorbance was observed in presence of OA. This laid a basis to envisage that under optimized conditions, the decrease in absorbance could have quantitative relation with concentration of OA. We first optimized the oxidant (Cr (VI)) to catalyst (Mn (II)) ratio by keeping contact time constant (30 min) and judiciously varying Cr (VI) and Mn (II) ratio while recording change in absorbance with respect each selected concentration of OA as shown in Figure 1 (B). The reaction was carried out in dark at 25 ºC. When fixing the oxidant and increase the catalyst from 1:0.25 to 1:0.5, the instrument can't detect small concentrations of oxalic acid such as 0.01mM, 0.1mM and 0.5mM. It can detect oxalic acid concentration greater than 1mM. However, 0.5:0.25 mM oxidant/catalyst ratio can detect small concentrations of oxalic acid and assured linearity of the calibration curve with correlation coefficient of 0.994. When diluting 0.5:0.25 oxidant/catalyst ratios to 0.05:0.025 oxidant/catalyst ratios the Cr (VI) reduction was finished after oxalic acid concentration of 0.1mM and it became the limiting reagent. That means in the reaction of Cr (VI) and Oxalic acid in the presence of Mn (II), the Cr (VI) reduced readily and the oxalic acid was remained alone. This resulted as concentration of oxalic acid increase; the absorbance reading became equal or stable. Since a better sensitivity (slope of the curve) quantitative response of the instrumental response (change in absorbance) to concentration of analyte was observed when the Cr (VI): Mn (II) ratio was 0.5:0.025, it was used as the best background ratio. This concentration ratio is consistence with previous results [20].

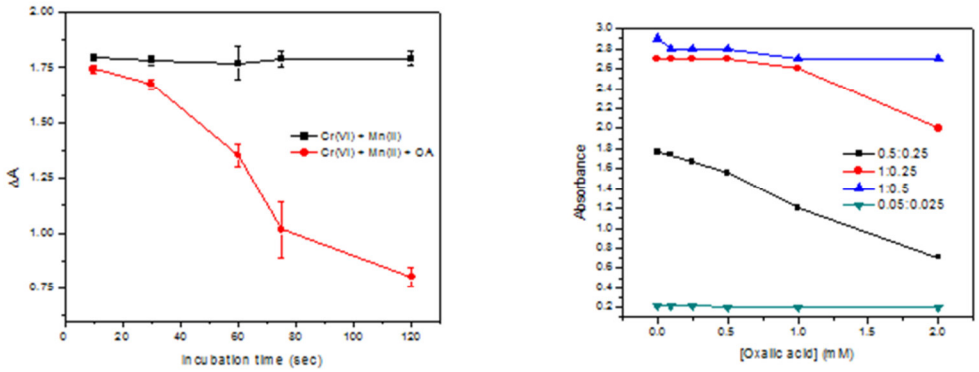

Figure 1 (A) Change in absorbance against different incubation time of background solution in absence (a) and presence of Oxalic acid (b); effect of (B) oxidant/catalyst ratio

Effect of reaction time

When oxalic acid was reacted with Cr (VI) in the presence of Mn (II), the reduction efficiency was depend on reaction time. So, fixing the reaction time that the oxidant can reduce and knowing completion time of the reaction was valid. After mixing same optimized combination of oxidant/catalyst with different concentrations OA, the reaction mixtures were incubated for 10, 30, 60, 90 and 120 minutes and absorbance measurements were performed at each time. The result is indicated in Figure 2. As can be seen in Figure 2 the reduction of Cr (VI) was related to the slope of calibration curve and the reaction time. As the reaction time increase, the Cr (VI) became reduced and the graph became sloppier. At 10 minute the absorbance result does not change while concentration of oxalic acid increased and it was less sloppy. After 30 minute the Cr (VI) starts to reduce (the absorbance became decreased to some extent) and the curve became sloppier than previous one. Even though, the calibration curve of all measurements showed reduction of Cr (VI), a more sensitive response was observed after 60 minute. Although a slight improvement in sensitivity could be observed at higher contact time, 60 min was chosen based on time economy.

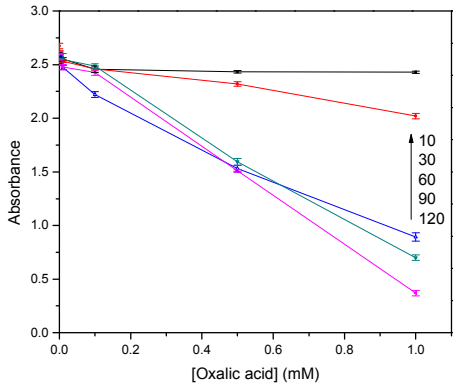

Figure 2. Effect of reaction time on reduction of Cr (VI) by OA

Effect of PH

In order to determine the best pH at which a faster reduction of Cr (VI) by OA can be observed, we carried out the reaction at previously optimized contact time and oxidant: catalyst ratio at pH 3, 5 and 9. The result is indicated in Figure 3.

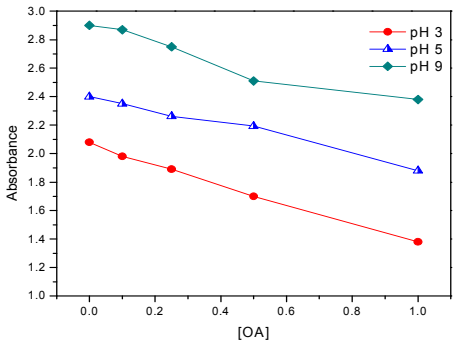

Figure 3. Effect of pH on reduction of Cr (VI) by oxalic acid

The best reduction of Cr (VI) was observed at PH 3 while oxalic acid concentration was increased. That means the reduction works in acidic media. Cr (VI) has slow reduction at slightly acidic conditions (pH 5). In basic media (pH 9) quantitative reduction of Cr (VI) was not observed. Therefore, pH 3 was selected.

Interference study

To study the selectivity of the proposed method, the effect of potential interfering substances that could exist in the studied fruit/vegetable samples were considered. For this purpose, maleic acid (MA) was considered because of its presence in most fruits and vegetables [21, 22]. The effect of MA on the absorbance of the Cr (VI) and OA mixture was studied by recording absorbance of Cr (VI) and OA mixture in presence and absence of MA. The presence of 1 mM MA could cause a difference of only 0.002 in absorbance. Due to this the developed method can be successfully applied for the detection of OA in the presence of other interfering species such as MA.

Table 1. The change in absorbance of chromium in absence and presence maleic acid

S/N	Cr (VI) (mM)	Mn (II) (mM)	OA (mM)	MA (mM)	ΔA
1	0.5	0.25	1	0	0.182
2	0.5	0.25	0	1	0.05
3	0.5	0.25	1	0.1	0.118
4	0.5	0.25	1	0.5	0.169
5	0.5	0.25	1	1	0.180

Determination of OA in real samples

Calibration curve of change in absorbance against standard concentrations of OA was constructed using 0.01, 0.25, 0.5, 1 and 2 mM. A linear relationship was observed between changes in absorbance of the background solution in presence of OA in the studied range with linear regression equation of $y=0.475x + 0.015$ with $R^2 = 0.997$ was recorded. Finally the amount of OA in the sample was determined from the calibration curve. The result is summarized in Table 2. The values of LOD and LOQ (n=5) were determined as the analyte concentration corresponding to 3 times of the standard deviation of the reagent blank divided by slope of the calibration curve (LOD) and 10 times standard deviation divided by the slope of the calibration curve (LOQ). Accordingly, the LOD and LOQ of the method were 0.0012 and 0.004 mM.

Table 2. Oxalic Acid concentration in selected dietary source samples (Mean ± S.D, n = 3)

S/N	Scientific name	Common name	Status	Abbreviated name	OA (mg/g)
1	*Brassica oleraceavar.acephala*	Ethiopian collared green	Raw	RCG	24.15 ± 0.565
			Cooked	CCG	12.05 ± 0.183
2	*Brassica oleracea Var. capitata*	Cabbage	Raw	RC	16.72 ± 0.388
			Cooked	CC	9.5 ± 0.424
3	*Lactuca sativa*	Lettuce	Raw	Le	14.44 ± 0.183
4	*Beta vulgaris*	Beetroot	Raw	Be	15.77 ± 0.175
5	*Ananas comosus*	Pineapple	Unripe	PBR	23.08 ± 0.212
			Fully ripe	PFR	18.31 ± 0.424
6	*Mangiferaindica*	Mango	Unripe	MBR	6.75 ± 0.212
			Fully ripe	MFR	1.18 ± 0.169

The concentrations of the OA along the fully ripe fruit sample showed a decrease in concentration as compared to unripe fruit sample and this might be the result of an increase in membrane permeability which allows acids to be stored in the respiring cells, formation of salts of OA, reduction in the amounts of acid translocate from the leaves, reduced ability of fruits to synthesize organic acids with fruit maturity, translocation

into sugars and dilution effect due to the increase in the volume of fruit as previously reported in the literature [23]. The high concentrations of OA in raw vegetables were also recorded as compared to values obtained from cooked vegetables. This indicated that cooking can reduce the level of OA in vegetable.

HPLC Chromatogram of OA standard for analyzing mango and Ethiopian collared green

Chromatogram of OA standard was recorded in order to determine the retention time that the chromatographic peak was eluted. The chromatographic peak for the OA standard for analyzing mango and Ethiopian collared green was eluted with a retention time of 2.039 and 7.485 minute respectively so the sample peak also expected to elute at this retention time. Experimental conditions are shown in Table 3.

Table 3. HPLC Chromatographic conditions (parameters) for analyzing OA standards

Chromatographic condition one		**Chromatographic condition two**
Column	Agilent poroshell-C18 250×4.6mm, 2.7µm connected series	Agilent poroshell-C18 100x2.1mm , 2.7 µm connected series
Mobile phase	50mM KH_2PO_4, PH 2.8 with H_3PO_4	50mMKH_2PO_4, pH 2.8 with H_3PO_4
Analysis Time	25 minute	25 minute
Flow rate	1ml/min.	0.6 ml/min.
Column Temperature	50°c	20°c
Injection Volume	5µl	5µl
Detector	UV with 210nm	UV with 210nm

Determination of OA in real samples using HPLC method

To determine the amount of OA in mango sample and Ethiopian collared green using HPLC method, calibration curve of OA standards were constructed under their perspective chromatographic condition. To determine the concentration of OA in mango sample, standard working serials of concentration of 0.1, 0.15, 0.2, 0.5 and 1 mg/ml in 2 N HCl were prepared and their observed peak areas were 164.342, 246.393, 330.555, 780.409 and 1509.965 respectively. Similarly to determine the concentration of OA in Ethiopian collared green, four standard working serials of concentration 0.1, 0.2, 0.5 and 1 mg/ml were prepared and the observed peak areas were 198.245, 444.323, 1000.592 and 1910.043 respectively. In this study OA standard solution was analyzed in triplicate and mean values were reported. The first chromatographic condition which was leveled as 'Chromatographic condition one' was used for analyzing the mango sample and the second chromatographic condition which was leveled as 'Chromatographic condition two' was used for analyzing the Ethiopian collared green. The standard curves were plotted to compute the regression equations and the concentration of oxalate from each sample solution was calculated from the equation of the corresponding standard curve. The results of the OA content indicated in Table 4.

Table 4. Total oxalic acid content of mango and Ethiopian collared green.

S/N	**Type of sample**	**Status**	**Abbreviated name**	**Total OA (mg/g)**
1	Ethiopian collared green*(Brassica oleraceavar.acephala)*	Raw	RCG	20.00
		Cooked	CCG	9.58
2	Mango*(Mangiferaindica)*	Fully ripe	MFR	0.74
		Before ripe	MBR	3.18

The analyzed green leafy vegetables and stony fruits in this study contained relatively low to moderate amounts of total oxalate that were less than 25 mg/g (Table 2 and Table 4). These results suggest that, the consumer should limit the use of these foods to an optimum per day [24]. However, the analyzed mango sample in this study is safe for consumption with little or no effect on the body system since it contains small amount of oxalate; less than 10 mg/g. In the present study, mangos were reported to contain 0.74 mg and 3.18 mg of total oxalate/g of dry weight for MFR and MBR, respectively. However, in the literature [25] the amount of oxalate in fully ripe mango was 1 mg/g of when analyzed with AOAC 1999 method and 1.07 mg/g of when analyzed with enzymatic method (Kit). The variation in oxalate values in different sources of plants can be affected by factors such as soil quality, climate condition and different state of fruit ripeness [26]. In addition, discrepancies could also be due to differences in preparation of the samples and analytical techniques. Interestingly, the level of OA was found to depend on the level of ripeness and cooking. Its level was higher in unripe and raw samples. The data in Table 4 is also reinforced by the corresponding decrease in the area under peak or intensities of the chromatograms (Figure 4). As can be seen in Figure 4, a more intense (higher area) peak was observed with unripe mango (Figure 4 (a) versus (b)) and raw vegetable (Figure 4 (c) versus (d)).

Percentage of oxalate loss due to cooking was calculated as 100-%TR (True Retention) [27].

$$\%\,TR = \frac{Oxalate\ content\ per\ g\ of\ cooked\ food\ \times g\ of\ food\ after\ cooking\ \times 100}{Oxalate\ coalntent\ per\ g\ of\ raw\ food \times g\ of\ food\ before\ cooking}$$

Therefore, the soluble oxalates in cabbage (GG) were reduced by 30.99% after boiling 5 min. However, the soluble oxalate levels in gurage gomen cooked (GGC) that was then boiled was 9.58 mg/g. These data suggest that household cooking is a very effective method to reduced soluble oxalate levels. Savage et al. (2000) [18] showed that boiling resulted in significant loss of soluble oxalates of some New Zealand foods such as spinach (Spinacia oleracea), silver beet (Beta vulgaris v.cicla) and rhubarb (Rheum rhaponticum).

Loss of oxalates in various vegetables is likely due to their leaching loss in cooking water. Other authors also reported a significant percentage loss of OA in different food items by cooking. Wanasundera and Ravindran (1992) [28] reported 40–50% loss of total oxalates when two kinds of yam tubers (Dioscoreaalata and D. esculenta) were boiled compared to steamed (20–25%). Judprason et al. (2006) [29] showed percentage loss ranging from 18% in coconut heart top stems (Cocos nucifera Linn.) to 76% in A. pennata. Therefore, the present findings are consistence with previous reports and reinforced the importance cooking as a strategy to reduce OA level in vegetables.

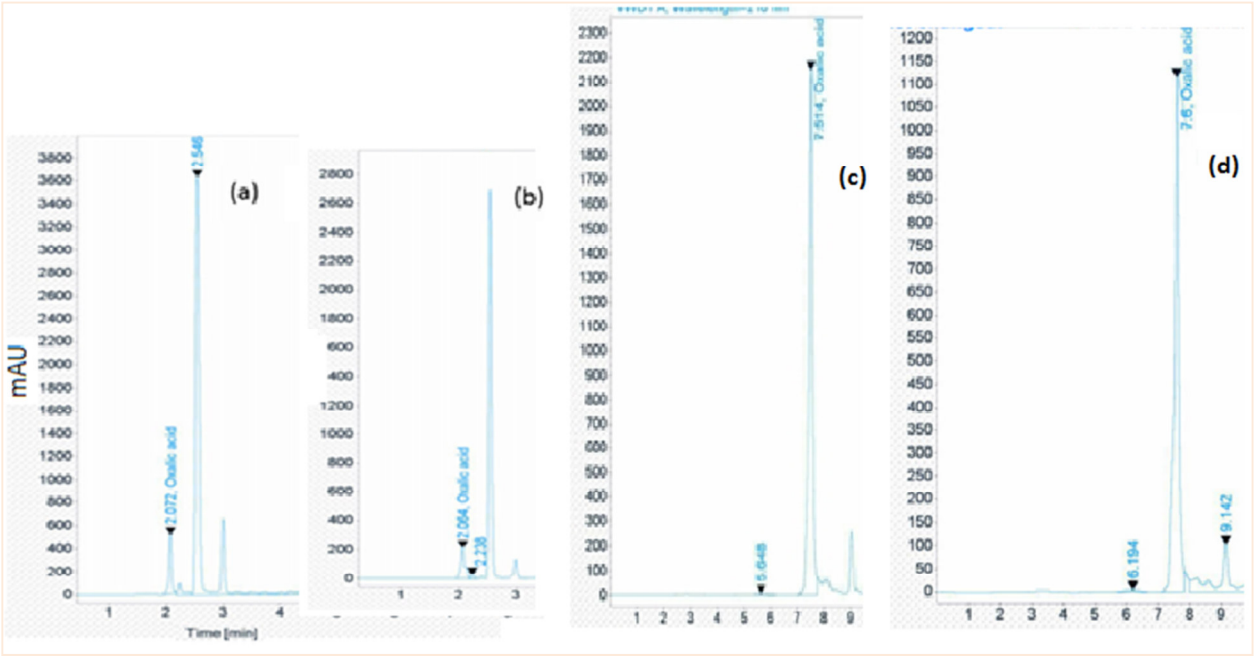

Figure 4. HPLC Chromatogram of (a) Mango Unripe and (b) Mango fully ripe, (c) Raw collared green cabbage and (d) Cooked collared green cabbage.

Comparison of the two methods

In the present study, the oxalate content that was analyzed by UV–Vis method yielded more total oxalate than HPLC method for both vegetables and fruit samples. The discrepancy of the results between the two methods might be due the difference of the extraction procedure, sensitivity of the analytical technique and effect of different experimental variables that we used for analysis. In both methods, the amount of total oxalate in cooked vegetable samples and fully ripe fruit samples were lower than raw vegetable samples and unripe fruit samples. Thus, the current method can fairly be used for the determination of OA in fruit and vegetable samples.

Conclusions

In this study, a new spectrophotometric method was successfully developed for the determination of oxalate content of selected food samples including mango and pineapple from stony fruits and beetroot, lettuce, Ethiopian collared green and cabbage from green leafy vegetables through catalytic titration with hexavalent chromium in presence of Mn (II) as a catalyst. A fairly good correlation was observed between the results from current method and standard method (HPLC). The studied samples contained relatively low to moderate amount of total oxalate (<25 mg/g dry weight). Out of ten foods studied, Ethiopian collared green *(Brassica oleraceavar.acephala)* contained highest concentration of oxalate, but it was reduced by 50 % after boiling for ten minute. Therefore, it can be concluded that the studied vegetable samples are safe for human consumption especially after cooking. The level of oxalate content in the analyzed fruit samples was found to decrease with increasing growing stage or state of ripeness. Fully ripe fruits contained lower concentration of oxalate than unripe fruits (at p=0.05, there is a significance difference between the fully and unripe fruits).

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