International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.2, pp 734-742, April-June 2014

Improvised Ferric reduction techniques for evaluating Anti-oxidant potential of ά-tocopherol and β-carotene

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Abstract: A couple of simplified procedures have been standardized to evaluate ά-tocopherol & β-carotene for their anti-oxidant potential by comparing their ferric ion reducing activity (FIRA). The techniques in general are capable of assaying agents in the range of 10 through 50 µg with perfect linearity showing FIRA in the range of 11 to 14 nmoles reduced per µg test agent using salicylate and thiocyanate assays. Methanolic ammonium thiocyanate protocol provides a very sensitive assay for alpha-tocopherol over 3 through 40 µg with FIRA values in the range of about 3 to 5 nmoles reduced per µg alpha-tocopherol. The study introduces an innovative protocol to conduct reduction process in organic solvents, and then to monitor reduction process in aqueous medium. This has enabled to demonstrate FIRA even with beta-carotene that so for has not been demonstrated successfully by other workers. An evaluation of treated samples with varying masses of beta-carotene and alpha-tocopherol has revealed that the test agents act by reduction and not by complexation since there was concentration related decrease in oxidized iron ($p<0.01$) while total iron in the samples remained consistently uniform ($p>0.1$). The present study has provided simplified approach to assay beta-carotene and alpha-tocopherol by ferric ion reducing activity, and obviated the interference if any by organic solvent soluble test nutraceuticals of pharmaceutical significance.
Key-words: Ferric reducing activity, Alpha-tocopherol, Beta-carotene, Salicylate, Thiocyanate, Assay.

Introduction

An important redox reaction employed for evaluating anti-oxidants is based on ability of test agents to cause reduction in ferric ion concentration. This ability has been variously labeled as ferric reducing anti-oxidant power (FRAP)[1-4], or ferric reducing power (FRP)[5] or ferric ion reducing activity (FIRA)[6]. The reaction is normally monitored using either Emmerie-Engel reaction for monitoring formation of mass of ferrous ions by ion-specific chelators forming colored complexes[7-10] or by using Trinder reaction that monitors reduction in ferric mass using salicylate reaction[6]. Whereas ά-tocopherol is easily assayable by these methods, β-carotene has failed to demonstrate ferric reducing ability by the employed methodologies[2,3]. The authors were encouraged by previous findings[6] and therefore intended to evaluate ferric thiocyanate as an alternative procedure to ferric-salicylate and to test both salicylate and thiocyanate for their suitability to evaluate β-carotene for its possible ferric reducing response. Since alpha-tocopherol and beta-carotene are soluble in organic solvents, it was quite feasible to conduct reduction and monitoring in organic solvents. However, in

view of the fact that target monitoring species, ferric ions are water soluble and solutions of beta-carotene are highly colored, an innovative protocol was envisaged to conduct reduction process in organic solvents, and then perform monitoring of reaction in aqueous medium. Consequently, experiments were designed to monitor response of alpha-tocopherol and beta-carotene under test conditions to salicylate and thiocyanate as monitoring reagents. Besides, micro-assay protocol for alpha-tocopherol was designed using ammonium thiocyanate method performed in organic solvents. Each reaction protocol was optimized for requirement of suitable experimental conditions. Besides, linearity checks for varying iron (III) estimations were conducted for each assay protocol.

Experimental

The experiments were carried out at an ambient temperature of 28.1 ± 0.6 ^{0}C. The drugs and chemicals used were of standard purity and quality obtained from reputed sources in India. Spectrophtometric measurements were made with UV-Visible Spectrophotometer, Model UVmini-1240 (Shimadzu Corporation, Japan).

Reagents

Alpha-tocopherol solution: Working solutions of α-tocopherol (Hi-Media) were prepared by dilution in methanol from stock solution of 1 % (w/v) alpha-tocopherol in ethanol.

Beta-carotene: The laboratory standard for beta-carotene was prepared from Antoxid capsules (Dr. Reddy's Labs). Each capsule with gross weight 469 ± 4 mg (n=11) contained 10 mg beta-carotene with minerals Zn sulfate (275 mg), Copper sulfate (1 mg Cu), Mn Sulfate (Mn 2 mg) and selenium dioxide (200 µg). The contents of 11 capsules were reconstituted in 50 mL chloroform, added 30 mL 2 M sodium chloride solution, well mixed. The chloroform phase was withdrawn in measured volume and filtered over Whatman Filter Paper 5, and filtrate made to volume with chloroform to contain 5 % ethanol. Working solutions were made in chloroform and standardized using absorption extinction coefficient at 465 nm[11].

Ferric chloride solution: The stock solution contained anhydrous ferric chloride as 25 µmole of iron (III) mL^{-1} 0.005 M HCl in water. Working solutions containing 1 and 2 µmole of iron (III) mL^{-1} were prepared by dilution of appropriate aliquot of stock solution in methanol at the time of use.

Sodium salicylate reagent: 3.2 %(w/v) sodium salicylate in water, 0.2M.

Sodium chloride solution: 11.7 % (w/v) NaCl in water, as 2 M.

Potassium thiocyanate solution: 10% (w/v) potassium thiocyanate in water.

Dilute HCl: 1 % and 10 % (v/v) HCl in water

Thioglycolic acid solution: 10 % (v/v) in water.

Dilute ammonia: 2 N ammonia made approximately from available stock solution of 25% ammonia in water.

Ammonium thiocyanate reagent: 5 %(w/v) in methanol.

Analytical methods

Monitoring ferric ion reducing activity in organic solvents

Ammonium thiocyanate assay for ά-tocopherol

Calibration curve for standard iron was prepared by taking iron (III) in 3.5 mL methanol as 0, 0.03, 0.06, 0.15 and 0.30 µmole, each was added 0.5 mL ammonium thiocyanate solution. The samples were allowed standing at room temperature for 25 to 30 minutes and then monitored for absorbance at 480 nm.

Based on the generated data, the assay for alpha-tocopherol was conducted at three iron concentrations: 0.1, 0.2 and 0.3 µmole. Each concentration was taken in one mL methanol, and added 1 mL methanol containing varying masses of ά-tocopherol found most suitable for the choice of iron mass: 3 through 15 µg with 0.1 µmole iron; 5 through 100 µg with 0.2 µmole iron and 10 through 80 µg with 0.3 µmole iron. The volume of each was made 4 mL with methanol and read at 480 nm following 25-30 minutes standing at room temperature. Decrease in absorbance with each concentration of test substance with respect to standard color in absence of the substance was computed, and net reduction in iron mass computed in nmoles.

Monitoring ferric ion reducing activity in aqueous phase

One milliliter methanol containing standard iron (III) mass (2 µmole) was added 1 mL methanol and 1 mL chloroform (optional while assaying alpha-tocopherol). For beta-carotene assay, test agent was in chloroform while for alpha-tocopherol it was in methanol in masses suitable for providing linear regression, usually 10 through 100 µg. The samples were allowed standing at room temperature for 30 minutes, and then added 5 mL 2M sodium chloride solution. The samples were mixed by gentle inversions and added color reagents, sodium salicylate or potassium thiocyanate, either directly or to the supernatants collected following centrifugation at about 8000 rpm for 5 minutes.

Thiocyanate method: Each mL supernatant was added 0.5 mL 10 % HCl, 1.5 mL water and 1 mL 10 % KSCN solution. The samples were mixed and monitored at 480 nm at 20-30 minutes. For direct conduct of assay, the reaction mixture following salination was added 1 mL 10 % HCl followed by 2.5 mL 10 % KSCN solution, and then centrifuged and assayed at 480 nm at 20-30 minutes.

Salicylate method: Three mL supernatant was added 1 mL 0.2 M sodium salicylate. The samples were allowed standing at room temperature for 40-50 minutes and monitored at 480 nm for absorbance. For direct conduct of assay, the reaction mixture was added 1 mL 0.2 M sodium salicylate reagent, and the color allowed developing for about an hour at room temperature and the supernatant monitored at 480 nm.

Calibration curve for iron was made using 0.2 through 1.2 µmole in 2 mL methanol and 1 mL chloroform, and following the procedure as outlined.

Determination of total and oxidized iron in supernatant

The supernatant aliquots from beta-carotene and alpha-tocopherol treated and untreated samples were subjected to analysis for total iron (reduced and oxidized) using a modification of thioglycollic acid[12]. Ferric iron has been monitored in the supernatants as per outlined salicylate and thiocyanate methods. For total iron determination, 1.5 mL supernatant was added 0.5 mL 1 % HCl followed by addition of 0.5 mL of 10% TGA reagent. Each sample was added 2 mL 3N ammonia, and absorbance monitored at 50-60 minutes at 535 nm. A linearity check to the TGA method was run using 0.1 through 1 µmole of iron (III).

Optimization studies

The assay techniques were optimized with respect to requirements for color-developing reagents in the different assay protocols and incubation period for reduction with respect to organic cum polar medium assay. Therefore, incubation time period optimization varied from 5 through 30 minutes using test agents beta-carotene 50 µg and alpha-tocopherol 100 µg incubated with 0.5 µmole iron (III) using thiocyanate/salicylate technique without centrifugation. Similarly the absorbance values of standard iron, 0.3 µmole, in absence and presence of 20 µg of alpha-tocopherol with chloroform 0, 0.5 and 1 mL was tested using ammonium thiocyanate assay. Optimal masses of the reagents were assessed by testing response of each assay over linear range of iron masses using two different concentrations chosen from trial experimentations. Reaction volume in each case was kept 4 mL. For salicylate assay 0.2 M sodium salicylate reagent was tested at 0.5 and 1 mL over 0.1 through 1.6 µmole iron (III). For aqueous thiocyanate assay, 10% KSCN solution was used as 1and 2 mL over 0.1 through 1.0 µmole iron (III) in about 0.1 M HCl. For ammonium thiocyanate assay optimization study, 5% ammonium solution was used at 0.5 and 1 mL while using 0.04 through 0.4 µmole iron (III).

Calculating anti-oxidant activity by ferric reduction

Regression analysis has been done by analyzing decrease in absorbance with treatments with respect to untreated standard vis-à-vis different masses of test substance used. Anti-oxidant potential has been assessed by performing regression analysis with respect to FIRP parameter that provides regression estimate *b* as FIRA value. The technique for calculating FIRP and FIRA have been already explained[6].

FIRP is an acronym for ferric ion reducing potential at a given mass of test substance. The parameter defines absolute mass of ferric ions in nmoles reduced by a given mass of test agent in microgram. FIRA is an acronym for ferric ion reducing activity. It defines mass of ferric ion as nmoles per sample reduced per microgram of the test material.. The calculations were conducted as follows:

(i) Calculate mass of iron in test sample in micromoles by test agent at its given concentration in micrograms:

$$C = [T/S]^* M$$

wherein

C= Estimated mass of iron in test sample, μmole

T= Absorbance value of standard iron in presence of test agent

S= Absorbance value of standard iron in absence of test agent

M=Mass of standard iron used, μmole

(ii) Calculate FIRP value for each test concentration:

. FIRP value, nmoles = $(M - C)^* 1000$

(iii) Perform regression analysis of FIRP values vis-à-vis mass of test substance used in micrograms. The estimated regression coefficient *b* indicates FIRA parameter. The same can be estimated directly for a given concentration as a coefficient of FIRP value in nmoles and mass of test substance in μg.

Results and Discussion

Methodologies

To enable performing ferric ion reduction assays for fat-soluble test agents such as alpha-tocopherol and beta-carotene, the available choice is always to perform the test in organic phase. Ferric salicylate method for assaying alpha-tocopherol in organic phase has already been developed by this laboratory[6]. Since ferric reduction can also be monitored with thiocyanate reagent, ammonium thiocyanate assay was mooted as suitable color reagent to monitor reduction in oxidized iron. This protocol worked with alpha-tocopherol because the solution of alpha-tocopherol in methanol provided colorless solution. The procedure failed to work with beta-carotene that yielded intensely colored solution in organic solvents interfering with measurements made at 480 nm. To enable assay of both alpha-tocopherol and beta-carotene based on ferric ion reduction, an improvised protocol was mooted so as to conduct reduction of iron (III) in non-polar phase, and conduct of determination and monitoring of extent of reduction in iron in polar phase. This procedure has offered two alternative approaches to conduct the assay. The color reagent could be added directly to the reaction mixture and colored aqueous phase withdrawn for measurements. Alternatively, the aqueous phase containing iron (III) can be separated out following centrifugation and reacted with color reagent. Two different color reagents can be used either salicylate (Trinder reaction) or thiocyanate to assay test agents by ability to reduce content of ferric ion. Use of sodium chloride solution was found to improve separation of phases and prevents ingress of chloroform into aqueous phase.

Table 1 Thiocyanate method for ά-tocopherol in methanolic medium

ά-tocopherol, µg	Observed Absorbance	Mean decrease absorbance	Mean reduction iron, nmole
0	0.171 ± 0.003	-	
3	0.146 ± 0.005	0.025	15
6	0.127 ±0.003	0.044	26
12	0.078 ± 0.002	0.093	54
15	0.048 ±0.002	0.123	72
Statistical features			
r ± S.E b± S.E		0.998±0.003 0.0082±0.0003	0.997.003 4.8 ± 0.2

[a] Standard response with iron, 0.1 µmole in absence of test agent

Ammonium thiocyanate assay for alpha-tocopherol

Ammonium thiocyanate assay was found to provide linear estimate over the concentration range of 0.03 through 0.3 µmole of iron (III) (r ± S.E. = 0.9999±0.0001; b ± S.E. = 2.16 ± 0.02, n=5 each). This enabled testing linearity at three different concentrations of iron. Alpha-tocopherol over the ranges was found to provide perfect linearity in deceasing absorbance and hence affecting reduction in iron (III). The data for 0.1 µmole iron (III) is given in Table 1 showing linearity in response over 3 through 15 µg (r ± S.E.= 0.998±0.003; b ± S.E. for absorbance decrease from control as 0.0082±0.0003; and for increase in FIRA in nmoles iron (III) reduced μg^{-1} as 4.8 ± 0.2, n=5 each). Mean pH during the assay remained 3.18 ± 0.03 (n=27). Similar responses were observed at 0.2 and 0.3 µmole iron (III) concentrations with following observations. At 0.2 µmole iron (III), 5 through 100 µg alpha-tocopherol exhibited a biphasic response: a steeper regression over 5 through 20 µg (r ± S.E.= 0.999±0.002; b ± S.E. for absorbance decrease from control as 0.0073±0.0002; and for increase in FIRA as 3.85 ± 0.12, n=5 each) while it was less steeper over 40 through 100 µg alpha-tocopherol (r ± S.E.= 0.996±0.005; b ± S.E. for increase in FIRA as 0.47±0.02, n=5 each). At 0.3 µmole iron, the data was linear over 10 through 40 µg alpha-tocopherol (r ± S.E. = 0.995±0.005; b ± S.E. for increase in FIRA as 2.8±0.2, n=5 each). As evident, the most effective linear ranges tend to increase with increase in mass of standard iron: as 3 to 15, 5 to 20 and 10 to 40 µg alpha-tocopherol, respectively, at 0.1, 0.2 and 0.3 µmole iron (III) while anti-oxidant potential in units of FIRA tend to decrease correspondingly from 4.78 at 0.1 µmole iron (III) to 3.85 at 0.2 µmole and then to 2.79 at 0.3 µmole iron (III).

Attempts to assay beta-carotene by this method did not work because beta-carotene even at concentration as low as 10 µg showed intense color and excessive absorbance at 480 nm. On the other hand, ammonium thiocyanate technique worked excellently with ascorbic acid with solutions made in aqueous phase showing perfect linearity over 5 through 20 µg (r ± S.E. = 0.996±0.005; b ± S.E. for increase in FIRA as 8.2 ± 0.4, n=4 each).

Aqueous thiocyanate method for alpha-tocopherol

The results are given in Table 2. As evident, alpha-tocopherol over 10 through 100 µg has shown linear response within 50 µg (r ± S.E. = 0.997±0.003; b ± S.E. for absorbance decrease from control as 0.00128±0.00006; and for increase in FIRA in nmoles iron (III) reduced μg^{-1} as 11.1 ± 0.50, n=5 each). The method employed incubation of test material with 2 µmole iron and then estimating reduction in iron in one milliliter of supernatant following centrifugation. A sharp decline in regression was noted by including data from 100 µg alpha-tocopherol. This necessitated taking separate experiment using 50, 75 and 100 µg alpha-tocopherol and 2 mL supernatant for assay. The assay revealed a less steep regression over the range (r ± S.E. = 0.996 ±0.004, b ± S.E. for FIRA values as 3.78 ± 0.02).

Aqueous thiocyanate method for beta-carotene: The assay was linear over 10 through 100 µg beta-carotene (Table 3) (r ± S.E. = 0.990±0.009; b ± S.E. for absorbance decrease from control as 0.00213±0.00013; and for increase in FIRA in nmoles iron (III) reduced μg^{-1} as 12.2±0.8, n=5 each). The assay employed centrifugation and addition of KSCN to supernatant (indirect method). Calibration curve for standard iron with thiocyanate in

aqueous medium under similar test conditions has revealed perfect linearity over 0.2 through 1.2 µmole iron (III) (r ± S.E. = 0.9992±0.0007; b ± S.E. = 0.697 ±0.013, n=6 each).

Aqueous salicylate method for alpha-tocopherol

The results are given in Table 4. As evident, alpha-tocopherol shows linear reduction in iron (III) over 5 through 100 µg (r ± S.E. = 0.9993±0.0006; b ± S.E. for absorbance decrease from control as 0.003804±0.00006; and for increase in FIRA in nmoles iron (III) reduced μg^{-1} as 13.4±0.2, n=5 each). The method utilized direct addition of color reagent to the reaction mixture. The mean pH during the assay was found to be 4.90 ±0.01 (n=15).

Aqueous salicylate method for beta-carotene: The results are given in Table 5. As evident, beta-carotene shows linear reduction in iron (III) over 5 through 100 µg (r ± S.E. = 0.9999±0.0001; b ± S.E. for absorbance decrease from control as 0.003193±0.00002; and for increase in FIRA in nmoles iron (III) reduced μg^{-1} as 14.0±0.1, n=6 each). The mean FIRA calculated directly for each concentration level yielded value 14.05 ± 0.30 comparable to that provided by regression analysis. The assay employed centrifugation and assay of supernatant for estimating reduction of iron (III). The similarly run iron showed perfect linearity over 0.2 through 1 µmole (r ± S.E. = 0.9997±0.0003; b ± S.E. = 0.383 ±0.004, n=5 each).

Aqueous salicylate method was found to provide linear estimate of ascorbic acid. Incubating 2 µmole of iron (III) with varying masses of ascorbic acid in total 4 mL reaction volume has revealed perfect linearity in iron reduction over 25 through 100 µg (r ± S.E. = 0.998±0.002; b ± S.E. for FIRA value as 7.5 ±0.3 n=4 each).

Table 2 Thiocyanate method for ά-tocopherol in aqueous medium

ά-tocopherol, µg	Observed Absorbance	Mean decrease absorbance	Mean reduction iron, nmole
0	0.231 ± 0.002[a]	Standard iron response in absence of test agent	
10	0.217 ± 0.002	0.014	121
30	0.188 ±0.002	0.043	372
50	0.166 ± 0.001	0.065	563
100	0.156 ±0.001	0.075	649[b]
Statistical features: linear range (10 through 50 µg)			
r ± S.E		0.997±0.003	
b± S.E		0.00128±0.00006	11.1±0.50

[a] Standard response with iron, 2 µmole in absence of test agent
[b] The value beyond linear regression
The values are mean ± S.E. of 5 observations each.

Table 3 Thiocyanate method for beta-carotene in aqueous medium

Beta-carotene, µg	Observed Absorbance	Mean ↓ Absorbance	Mean reduction iron, nmole
0	0.348±0.009[a]	Standard iron response in absence of test agent	
10	0.318±0.002	0.030	172
30	0.295±0.001	0.053	304
50	0.223±0.001	0.125	718
75	0.187±0.001	0.161	925
100	0.132± 0.002	0.216	1241
Statistical features			
r ± S.E		0.990±0.009	
b ± S.E		0.00213±0.00013	12.2±0.8

[a] Standard response with iron, 2 µmole in absence of test agent
The values are mean ± S.E. of 5 observations each.

Table 4 Salicylate assay for alpha-tocopherol in aqueous medium

Alpha-tocopherol, µg	Observed Absorbance	Mean ↓ Absorbance	Mean reduction iron, nmole
0	0.567±0.003[a]	Standard iron response in absence of test agent	
5	0.549±0.003	0.018	64
10	0.535±0.002	0.032	113
30	0.450±0.002	0.117	413
50	0.369± 0.002	0.198	698
100	0.191± 0.002	0.376	1326
Statistical features			
r ± S.E		0.9993±0.0006	
b ± S.E		0.003804±0.00006	13.4±0.2

[a] Standard response with iron, 2 µmole in absence of test agent
The values are mean ± S.E. of 5 observations each.

Table 5 Salicylate assay for beta-carotene in aqueous medium

Beta-carotene, µg	Observed Absorbance	↓ Absorbance	Mean reduction iron, nmole
0	0.459 ± 0.002[a]	Standard iron response in absence of test agent	
5	0.441 ±0.002	0.018	78
10	0.424 ± 0.001	0.035	147
30	0.361 ±0.002	0.098	428
50	0.298 ±0.004	0.161	703
100	0.137 ±0.002	0.322	1404
Statistical features			
r ± S.E	0.9999±0.0001		
b± S.E	0.003193±0.00002	14.0±0.1	

[a] Standard response with iron, 2 µmole in absence of test agent
The values are mean ± S.E. of 6 observations each.

Determination of total iron with thioglycollic acid

It was felt necessary to rule out the possibility that iron decrease was not due to any complexation process but due to reduction. This necessitated assaying iron total with respect to oxidized form of iron. Both thiocyanate and salicylate techniques measure oxidized form of iron while thioglycollic assay technique measures total iron. Hanzal's method[12] was employed to determine total iron content in beta-carotene and alpha-tocopherol treated samples containing 2 µmoles of iron (III). The method measures both ferrous and ferric iron compared to salicylate or SCN methods that are selective for oxidized form of iron (III).

Mean absorbance values for total iron with varying masses of beta-carotene or alpha-tocopherol did not differ from those of untreated samples ($p>0.1$, n= 4 each). For instance, the values with 1 mL aliquots of supernatants with alpha-tocopherol 10, 30 and 50 µg have remained respectively as 0.132 ± 0.001, 0.133 ± 0.001 and 0.136 ± 0.003 compared to that of untreated sample as 0.136 ± 0.001. This indicated an overall mean recovery of 1.96 µmole (ca. 98 % recovery) while thiocyanate assay that measured only iron (III) provided respective mean absorbance values as 0.217 ± 0.002, 0.188 ±0.002 and 0.166 ± 0.001 compared to untreated value 0.231 ± 0.002 showing concentration related fall in absorbance with perfect linearity (r± S.E. = 0.997 ± 0.003, and reduction in FIRA as 11.0 ± 0.5 nmoles per µg). Similar observations were seen with beta-carotene. The mean absorbance for total iron at 30, 50 and 100 µg has remained respectively as 0.181 ± 0.007, 0.176 ± 0.003 and 0.170 ± 0.008 with respect to untreated sample as 0.179 ± 0.006 ($p>0.1$, n = 4 each) with overall recovery of 1.97 µmole (98.3 % of untreated) while absorbance values for oxidized iron (by salicylate method) were found to be as 0.267 ± 0.006, 0.209 ±0.008 and 0.114 ± 0.006 compared to untreated absorbance value 0.324 ±0.009

indicating concentration related significant fall in iron (III) ($p<0.01$, n= 4 each) with perfect linearity (r± S.E. = 0.995 ± 0.006, and reduction in FIRA as 13.2 ± 0.8 nmoles per µg). These observations made on random samples are consistent with the results on alpha-tocopherol and beta-carotene on reduction of iron (III) as evidenced by the current studies. The results have confirmed that beta-carotene and alpha-tocopherol act by reducing iron (III) and any non-specific complexation as cause of reduction in iron content is ruled out since total iron remains same in each sample while oxidized form is quantitatively reduced. Under current setting, TGA assay for total iron was found to be linear over 0.1 through 1 µmole iron in 4 mL reaction volume (r ± S.E, 0.9999 ±0.0001, b± S.E, 0.7572 ±0.0001, n=4 each).

Optimization studies

Optimization experiments were required to optimize requirement rates of reagents, and to optimize incubation time period necessary for conduct of assay. With either test agent, reduction improved with increase in incubation period. With alpha-tocopherol, mean percent reduction in iron was found to increase from about 71 % at 5 min to 85 % at 30 minute with corresponding absorbance values recorded as 0.057 ± 0.001 and 0.029 ± 0.001 compared to untreated standard value 0.197 ± 0.001 ($p <0.01$, n= 6 each). Similar trend was observed with beta-carotene, showing corresponding absorbance values as 0.029 ± 0.001 and 0.006 ± 0.001 indicating reduction has improved from about 86 % at 5 minute to 97 % at 30 minute ($p <0.01$, n= 6 each). This allowed selection of 30 minute as appropriate incubation time for the assay.

Chloroform usage is optional while conducting alpha-tocopherol assay. Chloroform at 0.5 and 1 mL did not affect the reduction action of alpha-tocopherol. The mean absorbance values were respectively recorded as 0.378 ± 0.004 and 0.386 ± 0.004 compared to the value without chloroform as 0.381 ± 0.002 ($p>0.1$, n = 3 each) indicating per cent reduction from untreated value 0.578 ± 0.006 33 to 35 per cent ($p<0.01$).

Optimal mass of ammonium thiocyanate for ammonium thiocyanate assay was found to be 25 mg while that of potassium thiocyanate in aqueous assay in presence of about 0.1 M HCl was found to be 100 mg in 4 mL volume. Mass of sodium salicylate for conducting salicylate method in non-acidic medium was found to be 32 mg, corresponding to 1 mL 0.2 M sodium salicylate. Increase in mass of reagents has improved regression by about 3 to 16 percent. Ammonium thiocyanate at 25 and 50 mg has increased regression coefficient from 1.59 ±0.02 to 1.63 ± 0.05 showing about 3 % increase. Potassium thiocyanate, 100 mg and 200 mg in presence of about 0.1 M HCl acidity has increased regression from 1.64 ± 0.02 to 1.90 ±0.01 with about 16 % increase while sodium salicylate at 16 and 32 mg has improved regression by about 13 % from 0.420 ± 0.008 and 0.473 ±0.004. Absorbance maxima for salicylate assay in organic phase using salicylic acid in methanol[6] has been found to be 525 nm (regression estimate at 525 nm was 1.4 times that at 480 nm) while that of sodium salicylate in aqueous medium was found to be 480 nm with regression estimate 1.3 times the value found at 525 nm.

Random checks on pH following each assay has revealed that mean pH with ammonium thiocyanate, potassium thiocyanate, and sodium salicylate assay systems have remained respectively as 3.30 ± 0.04 (n=9), 2.70 ± 0.03 (n=20) and 4.90 ± 0.01 (n=32). Mean pH during thioglycollic acid assay has remained 10.72 ± 0.02 (n=10). Thiocyanate assay is appropriately conducted while maintaining 0.01 through 0.5 M acidity. Ammonium thiocyanate per se maintained adequate acidic pH for conduct of the assay while it was ensured by addition of dilute HCl while conducting potassium thiocyanate assay. Increasing acidity with addition of 0.1 through 1 mL 1 % HCl was found to enhance absorbance values further with ammonium thiocyanate assay and a perfect correlation existed between decrease in pH from 4.9 to 1.95 with increase in absorbance from 0.33 to 0.73 (r= -0.992). However, no attempt was made to make any incremental change in basal acidity provided by ammonium thiocyanate. High acidity was found to cause decolorization of ferric-salicylate, and color returned back with addition of alkali. A 4 mL reaction volume was found to tolerate 0.1 mL of 1 % HCl without any adverse effect on absorbance ($p>0.1$, n= 5 each) while higher concentrations (0.3 and 1 ml 1% HCl caused concentration related decrease in absorbance ($p<0.01$, n= 5 each). Therefore, presence of acidity should be avoided or minimized while conducting the test.

Conclusions

A couple of inexpensive simple photometric methods have been provided to enable assaying anti-oxidant activity of alpha-tocopherol and beta-carotene. Two versions of assays have been developed depending upon nature of reaction medium. The assay in organic solvent is applicable to test agents such as alpha-tocopherol providing almost colorless solution in organic solvents. Another version is an innovative procedure that enables conduct of reduction in organic phase and monitoring reduction in aqueous phase. The assay is applicable to both colorless as well as colored test agents using either thiocyanate or salicylate as monitoring reagent for ferric ion. The procedure has enabled demonstration of ferric ion reducing power in alpha-tocopherol and beta-carotene. Ferric reducing ability in beta-carotene has not been so for successfully demonstrated by other workers[2,3]. The improvised protocol has been extensively found handy and useful to screen chloroform extracts from Indigenous medicinal plants for their anti-oxidant evaluation (unpublished).

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