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Interaction of Oilseed Pigments and Phospholipids in the Determination of Total Phenolic Compounds using the Folin-Ciocalteu Reagent

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Abstract: A study was done to test the effect of minor components in vegetable oils on the accuracy of the method used for the determination of total phenolic compounds in vegetable oils using the Folin-Ciocalteu reagent (F-C). Main minor components in vegetable oils include chlorophyll, carotenoids and phospholipids and they were all considered in this study. Extracts of chlorophyll as well as carotenoids were prepared from rocket and carrot, respectively. Those extracts as well as a commercial sample of lecithin as being a common phospholipid in vegetable oils were added at different concentrations to a refined, bleached and deodorized sunflower oil (SFO). These oil blends were then subjected to spectrophotometric determination of total phenolic compounds using Folin-Ciocalteu reagent (F-C). The results of this study have shown that the effect of carotenoids and lecithin on the absorbance of the reaction product of phenolic compounds with Folin-Ciocalteu reagent at 765 nm used for the estimation of total phenolics in vegetable oils is negligible while the reverse was true in case of chlorophyll. As the chlorophyll concentration in the oil increases, the absorbance of the reaction product of phenolic compounds with Folin-Ciocalteu reagent at 765 nm increases and the estimated correlation coefficient, R^2, was found to be 0.95. This indicates that chlorophyll has a considerable reactivity towards the Folin-Ciocalteu reagent used giving higher estimates of phenolic content than the actual values. This suggests that this method is not a reliable one for the estimation of total phenolics in case of oils rich in chlorophyll if conventional procedure was followed. However, an equation has been derived using the results of this work that can be used for a more accurate estimation of phenolic compounds using Folin-Ciocalteu reagent by exclusion of the effect of chlorophyll interaction. By using this equation, the actual phenolic content in two olive oil samples used in this study would be 14 and 11% less than the estimated values following conventional procedure.
Key words: F-C Reagent, Carotenoids, Chlorophyll, Phospholipid, Total Phenolics.

1 Introduction

The Folin-Ciocalteu (F-C) reagent which consists of a mixture of sodium molybdate, sodium tungstate and other reagents can be used for the estimation of total phenolic compounds in food components such as edible vegetable oils[1]. The reaction of phenolic compounds with this reagent is considered as an oxidation/reduction reaction in which the phenolic group is oxidized and the molybdenum and tungsten ion is reduced[2]. Upon reaction of this reagent with phenols, it produces a blue colour which absorbs at 765 nm which is thought to be due to a complexed Mo(V) species[3]. Over the years, there have been numerous reports that

proteins, carbohydrates, amino acids, nucleotides, thiols, unsaturated fatty acids, vitamins, amines, aldehydes and ketones may contribute to this reagent[1,4,5,6]. It has been reported in a valuable review that large number of substances react with F-C reagent and the extent to which those substances may react with F-C reagent has been also discussed in this review[4]. Those substances include tertiary aliphatic amines, hydrazine, certain purines, tryptophan, hydroxylamine, and other organic and inorganic reducing agents. It was reported that other compounds that may be found in plant food extracts as reducing sugars and ascorbic acid can be also reactive to F-C reagent and accordingly, the results of determination of total phenolic compounds (TPC) will be inaccurate[6,7,8,9]. It has been also reported[1] that the F-C method suffers from many other interfering substances such as sugars, aromatic amines, sulfur dioxide, ascorbic acid (AA) organic acids and a correction for these substances should be made. Also, some inorganic substances may be reactive to F-C reagent to give elevated apparent phenolic contents[4,5].

In the same scope, the reactivity of the F-C reagent towards nitrogenous compounds have been determined[10]. These compounds cover several chemical classes and it was found that most of them have considerable reactivity towards the F-C reagent. Examples of these classes are guanidines, tertiary amines, hydrazines, hydroxylamines, aromatic amines, pyrroles and indoles. Since some compounds in these classes occur in plants, it may be that measurement of phenols by the F-C method could possibly give too high of estimates of phenolic content. It has been also reported that the correlation coefficient between the phenolic content of olive oil as determined by HPLC method and that determined by F-C reagent was a moderate one ($r = 0.64$)[11]. The case of determination of catechins in chocolate is an example of this [12].

Edible vegetable oils vary widely in their content of some specific components such as phospholipids and oilseed pigments. Carotenoids and chlorophyll are two major pigments which occur naturally in oilseed and are present as minor components in edible vegetable oils. Although of the extensive research work that has been carried out to determine the reactivity of the F-C reagent towards several classes of compounds, nothing is reported so far about the reactivity of this reagent towards oilseed pigments and phospholipids. Interference of these components with the reagent may give false results in concern of the estimation of phenolic content using the Folin-Ciocalteu (F-C) assay.

The purpose of the present work is to investigate whether carotenoids, chlorophyll or phospholipids are reactive with the Folin-Ciocalteu (F-C) reagent and how it would affect the reliability of this method for the estimation of total phenolics in vegetable oils.

2 Materials and Methods

2.1 Materials

Fresh carrot roots (*Daucus carota*) and fresh leaves of green rocket plant (*Eruca sativa*) were purchased from local market in August 2015 for the preparation of the carotenoids and chlorophyll extracts respectively. Sunflower oil (SFO) used was of an edible grade and was purchased from local market. Two samples of extra virgin oil were used in this study. These olive oil samples were extracted from olives, collected from two different farms in Egypt in two different locations. The first is Wadi Food farm located in the desert near Alexandria Cairo Desert Road while the other farm was in Sinai Desert. Folin and Ciocalteu's Phenol Reagent (FCP reagent) was purchased from Sisco Research Laboratories Pt. Ltd.. Lecithin (purity ≥ 97%) was purchased from für die Biochemie, ROTH, Carl Roth GmbH + Co. KG. All other chemical reagents used were of analytical grade and were purchased from Fisher Scientific, UK.

2.2 Methods

Carotenoids were extracted from carrot roots and chlorophyll pigment was extracted from rocket plant according to the standard methods[13]. The two extracts were then used to prepare two groups of samples of sunflower oil; one group is enriched with carotenoids while the other was enriched with chlorophyll.

2.3 Extraction of carotenoids and preparation of SFO samples enriched with carotenoids

The fresh carrot roots were thoroughly washed, cut into small pieces and was then ground in a food chopper. To the ground carrot roots, 2 g $MgCO_3$ was added for each 100 g of the carrot roots and was

vigorously shook with a mixture of acetone and n-hexane (1:1, v/v) for five minutes. The whole mixture was allowed to settle down and the solvent mixture was filtered off. The extraction procedure was repeated three times and the collected three extracts were washed with distilled water to remove acetone from the solution of carotenoids in hexane. The hexane was then completely evaporated at 40°C under vacuum yielding the extracted carotenoids.

The extracted carotenoids were then added to 100 mL sunflower oil to give a sample of sunflower oil rich in carotenoids. The latter sample was then used to prepare several other oil samples of sunflower oil containing different concentrations of carotenoids.

2.4 Extraction of chlorophyll and preparation of SFO samples enriched with chlorophyll

The fresh leaves of rocket were thoroughly washed with water, cut into small pieces and ground in a food chopper then two gram of Na_2CO_3 was added to each 100 g of the ground rocket leaves. The ground material was stirred with a mixture of acetone/water (85:15, v/v) for five minutes and the liquid extract was then filtered off. The extraction procedure was repeated three times and the acetone of the total of the three extracts was evaporated under vacuum at 40°C to get the chlorophyll pigment.

As followed in case of carotenoids, the extracted chlorophyll was added to 100 mL sunflower oil to give a sample of sunflower oil rich in chlorophyll. The absorbance of this sample was then recorded at 670 nm using UV-visible spectrophotometer (UV-160 1PC, UV-visible spectrophotometer, Shimadzu, Tokyo, Japan). This wave length is the one specific of chlorophyll[14] and this reading at this wave length is proportional to the chlorophyll content. That sample which was rich in chlorophyll was then used to prepare several other oil samples of sunflower oil containing different concentrations of chlorophyll.

2.5 Preparation of oil samples containing different concentrations of lecithin as a common phospholipid in most vegetable oils

Three grams of lecithin powder was dissolved in n-hexane, added to hundred milliliters of sunflower oil and n-hexane was then evaporated from the mixture under vacuum. The produced sunflower oil sample which was rich in lecithin was then used to prepare several oil samples of sunflower oil containing different concentrations of lecithin.

2.6 Isolation of total phenolics

Sunflower oil and samples of this oil enriched with chlorophyll as well as those enriched with carotenoids and lecithin were subjected to extraction procedure to isolate their phenolic compounds[15]. Briefly, 10 mL of each oil sample was dissolved in 10 mL n.hexane followed by the addition of 10 mL of MeOH/H_2O mixture (70:30, v/v) and the mixture was shook thoroughly. Two separate liquid phases have been formed; a non polar one consisting of the non-phenolic components dissolved in n-hexane and a polar one containing the phenolic compounds dissolved in MeOH/H_2O mixture. The extraction procedure was repeated three times using ten mL of MeOH/H_2O mixture each time as to recover all phenolic compounds.

In addition to sunflower oil samples, the phenolic compounds in two oil samples of olive oil were similarly isolated; one extracted from olives of Wadi Food farm while the other extracted from the olives of a farm in Sinai Desert. The chlorophyll concentration of each olive oil sample was then estimated according to its absorbance at 670 nm compared to that of the chlorophyll - rich sunflower oil sample previously prepared.

2.7 Determination of total phenolics content

The determination of the total phenolic content in the isolated phenolic extracts from sunflower oil and olive oil, as previously mentioned, has been made following the standard method using Folin-Ciocalteu (F-C) reagent with slight modification[16,17]. Two aliquots, (100 µL each) were taken from each oil sample, put in two test tubes and the liquid volume in each was made up to three milliliters using distilled water. Two milliliters of 10-fold diluted F-C reagent was then added to each test tube and after five minutes one mL of sodium carbonate solution (7.5% concentration) was added. After thirty minutes, the absorbance of the whole mixture was measured at 765 nm using a UV-visible spectrophotometer (UV-160 1PC, UV-visible spectrophotometer, Shimadzu, Tokyo, Japan). A blank was used which have been subjected to all previously mentioned procedure using same reagents with exclusion of the phenolic extract.

3 Results and discussion

The effects of the concentration of carotenoids, lecithin and chlorophyll in sunflower oil on the absorbance reading at 765 nm of the reaction product of phenolic compounds present in that oil with F-C reagent are shown in Tables (1-3). As pre-mentioned, the concentration of phenolic compounds in oil is directly proportional to the absorbance of its reaction product with F-C reagent at 765 nm. The data listed in these tables are graphically represented in Figures (1-3) in which the concentration of carotenoids, lecithin and chlorophyll in sunflower oil, g/100 mL oil (represented on the X axis) are plotted versus the absorbance reading at 765 nm (represented on the Y axis). It is clear from Figures (1 and 2) that the data showing the effect of the variations in the oil content of each of carotenoids as well as lecithin are quite scattered with no apparent relation that can be observed. The correlation coefficient R^2 of the data presented in these two figures have been estimated and found to be very low being 0.2556 in case of carotenoids and 0.1766 in case of lecithin indicating a very weak relationship. This means that the interference of carotenoids as well as lecithin in the F-C reaction is quite negligible. However, the reverse was true in case of chlorophyll. The data listed in Table (3) show the variations in the absorbance of the reaction product of phenolic compounds in sunflower oil with Folin-Ciocalteu reagent at 765 nm due to the variations of the concentration of chlorophyll. It is quite clear that the absorbance reading gradually increases with the increase of chlorophyll concentration. The data are graphically represented in Figure (3) which shows that there is a strong linear relationship between the chlorophyll concentration (on the X axis) and the absorbance at 765 nm (on the Y axis) (correlation coefficient R^2 = 0.9531). The predicted equation that relates, the absorbance of the reaction product with F-C reagent at 765 nm, (Y) with the concentration of chlorophyll, g/100 mL oil, (X) is as follows:

$$Y = 0.7828\ X + 0.0062 \quad \text{(correlation coefficient, } R^2 = 0.953\text{)}$$

Table (1): Absorbance of the reaction product of phenolic extracts from sunflower oil samples having different concentrations of carotenoids with Folin-Ciocalteu Reagent at 765 nm.

Concentration of carotenoids in the oil sample, g/100 mL oil	Absorbance of the reaction product with Folin-Ciocalteu Reagent at 765 nm
0.003	0.012
0.008	0.026
0.009	0.016
0.011	0.017
0.018	0.019
0.021	0.016
0.048	0.013
0.076	0.023
0.079	0.027
0.082	0.033
0.085	0.028
0.088	0.018
0.089	0.018

Table (2): Absorbance of the reaction product of phenolic extracts from sunflower oil samples having different concentrations of lecithin with Folin-Ciocalteu Reagent at 765 nm.

Concentration of lecithin in the oil sample, g/100 mL oil	Absorbance of the reaction product with Folin-Ciocalteu Reagent at 765 nm
0.38	0.101
0.75	0.153
1.13	0.191
1.5	0.139
1.87	0.095
2.25	0.08

Table (3): Absorbance of the reaction product of phenolic extracts from sunflower oil samples having different concentrations of chlorophyll with Folin-Ciocalteu Reagent at 765 nm.

Concentration of chlorophyll in the oil sample, g/100 mL oil	Absorbance of the reaction product with Folin-Ciocalteu Reagent at 765 nm
0.0002	0.012
0.0166	0.023
0.0217	0.024
0.0244	0.02
0.0443	0.058
0.0459	0.042
0.0461	0.031
0.0538	0.035
0.0595	0.051
0.0905	0.074
0.1336	0.122
0.17	0.136

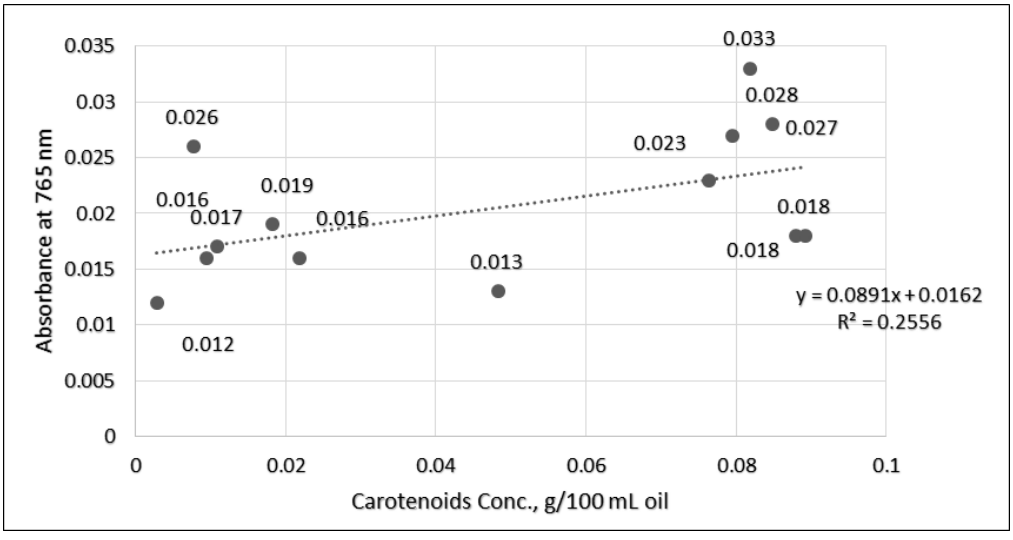

Figure (1): Effect of carotenoids concentration in sunflower oil on the absorbance of the reaction product of the phenolic extracts from oil with Folin-Ciocalteu Reagent at 765 nm.

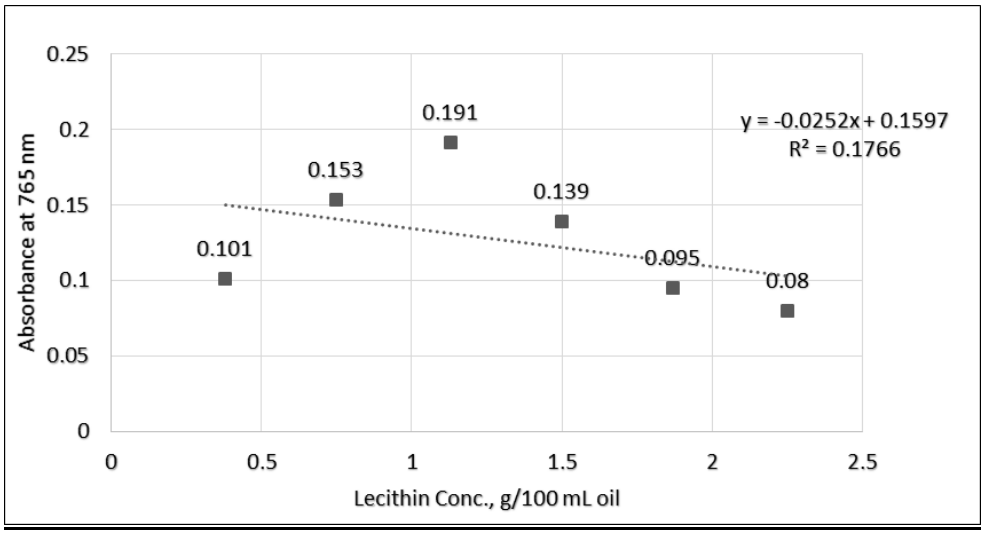

Figure (2): Effect of lecithin concentration in sunflower oil on the absorbance of the reaction product of the phenolic extracts from oil with Folin-Ciocalteu Reagent at 765 nm.

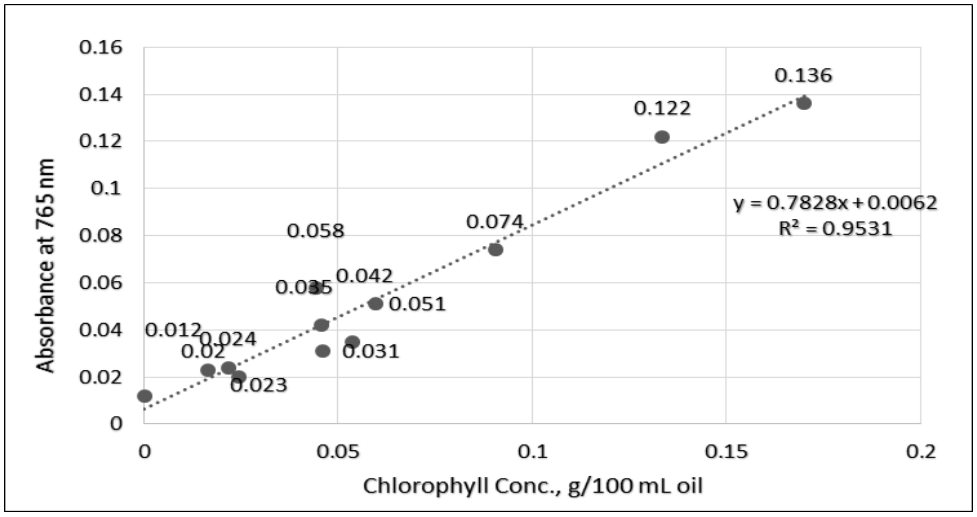

Figure (3): Effect of chlorophyll concentration in sunflower oil on the absorbance of the reaction product of the phenolic extracts from oil with Folin-Ciocalteu Reagent at 765 nm.

This means that chlorophyll interacts with Folin-Ciocalteu reagent resulting in an increase in the absorbance reading at 765 nm giving false over estimation of the concentration of phenolic compounds. Therefore, it is highly recommended to account for the effect of the presence of chlorophyll whenever an estimate of phenolic compounds is to be measured using F-C reagent.

The previously mentioned equation has been utilized in case of the two olive oil samples considered in this study whereby an account has been made for the effect of chlorophyll on the absorbance reading at 765 nm. Tables (4) and (5) show a list of the absorbance readings at 765 nm, the concentration of chlorophyll, g/100 mL oil, the absorbance at 765 nm due to chlorophyll interaction with F-C reagent using the derived equation and the corrected absorbance reading after exclusion of the effect of chlorophyll. According to the listed results, the percentage increase in the absorbance at 765 nm due to chlorophyll has been estimated and found to be 14% and 11% in case of olive oil from Wadi Food farm and Sinai Desert farm respectively. This, in turn, means an over estimation of phenolic compounds of the two oils by the same percentages.

Table (4): Results of phenolic estimation in olive oil extracted from olives in Wadi Food farm.

Absorbance at 765 nm after reaction with F-C reagent	0.407
Conc. of chlorophyll, g/100 mL oil	0.0555
Absorbance at 765 nm due to chlorophyll estimated using the derived equation	0.0493
Corrected absorbance of the reaction product with F-C reagent at 765 nm by exclusion of the effect of the chlorophyll	0.3577
Percentage increase in absorbance due to chlorophyll (Percentage over estimation of phenolic compounds)	14.00

Table (5): Results of phenolic estimation in olive oil extracted from olives in Sinai Desert farm.

Absorbance at 765 nm after reaction with F-C reagent	0.315
Conc. of chlorophyll, g/100 mL oil	0.0319
Absorbance at 765 nm due to chlorophyll estimated using the derived equation	0.0313
Corrected absorbance of the reaction product with F-C reagent at 765 nm by exclusion of the effect of the chlorophyll	0.2837
Percentage increase in absorbance due to chlorophyll (Percentage over estimation of phenolic compounds)	11.00

4. Conclusion

It is concluded that chlorophyll interacts with Folin-Ciocalteu reagent used for the determination of phenolic content so that it would give higher absorbance reading at 765 nm and hence higher estimates of phenolic compounds than the actual values. This suggests that this method is not a reliable one for the estimation of total phenolics in case of oils rich in chlorophyll if conventional procedure was followed. However, the equation that has been derived using the results of this work can be used for a more accurate estimation of phenolic compounds using Folin-Ciocalteu reagent by exclusion of the effect of chlorophyll interaction.

References

1. Prior RL, Wu X and Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem., 2005, 53: 4290–4302.
2. Agbor, GA, Vinson JA and Donnelly PE. Folin-Ciocalteau reagent for polyphenolic assay. International Journal of Food Science, Nutrition and Dietetics (IJFS), 2014, 3: 147-156.
3. Singleton VL and Rossi Jr J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic., 1965, 16: 144–158.
4. Peterson GL. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal. Biochem., 1979, 100: 201-220.
5. Box JD. Investigation of the Folin-Ciocalteu phenol reagent for the determination of polyphenolic substances in natural waters. Water Res., 1983, 17: 511-522.
6. Everette JD, Bryant QM, Green AM, Abbey YA, Wangila GW and Walker RB. Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. J. Agric. Food Chem., 2010, 58: 8139–8144.
7. Singleton VL, Orthofer R and Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu Reagent. Methods Enzymol., 1999, 299: 152-178.

8. Huang D, Ou B and Prior RL. The chemistry behind antioxidant capacity assays. J. Agric. Food Chem., 2005, 53: 1841-1856.
9. Sánchez-Rangel JC, Benavides J, Heredia JB, Cisneros-Zevallos L and Jacobo-Velázquez DA. The Folin–Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. Anal. Methods, 2013, 5: 5990-599.
10. Ikawa M, Schaper TD, Dollard CA and Sasner JJ. Utilization of Folin-Ciocalteu reagent for the detection of certain nitrogen compounds. J. Agric. Food Chem., 2003, 51: 1811–1815.
11. Andjelkovic M, Van Camp J, Pedra M, Renders K, Socaciu C and Verhe´ R. Correlations of the phenolic compounds and the phenolic content in some Spanish and French olive oils. J. Agric. Food Chem., 2008, 56: 5181–5187.
12. Mursu J, Voutilainen S, Nurmi T, Tuomainen T-P, Kurl S and Salonen JT. Flavonoid intake and the risk of ischaemic stroke and CVD mortality in middle-aged Finnish men: the Kuopio Ischaemic Heart Disease Risk Factor Study. Br. J. Nutr., 2008, 100: 890-895.
13. AOCS. Official Methods of Analysis of AOAC International, 19th Edition, Ed. Jr., G.W.M, Published by AOAC International, vol. I and II, 2012.
14. El-Noamany HM, Megahed OA and Zaher FA. Comparison between different types of common adsorbents: Part II: Adsorption capacity to remove carotenoids as compared to chlorophyll pigment. TESCE, 2014, 40: 16-28.
15. Tasioula-Margari M and Okogeri O. Isolation and characterization of virgin olive oil phenolic compounds by HPLC/UV and GC-MS. Journal of food science, 2001, 66: 530-534.
16. Singh RP, Murthy KNC and Jayaprakasha GK. Studies on antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. J. Agric. Food Chem., 2002, 50: 81–86.
17. El-Hamidi M and El-Shami SM. Scavenging activity of different garlic extracts and garlic powder and their antioxidant effect on heated sunflower oil. American Journal of Food Technology, 2015, 10: 135-146.

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