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IS QUERCETIN ACCUMULATING IN EGGS USING FLAVONOIDS-ENRICHED POULTARY FEED?

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ABSTRACT

Flavonoids-enriched feed, besides enhancing poultry performance like increase in meat's shelf life, egg laying rate, egg shell strength, protein contents and Haugh unit and decrease in fats contents of yolk, may result in accumulation of active components such as quercetin in eggs, which has not yet verified. Therefore, the present study describes a simple ultraviolet spectrophotometric method for the determination of quercetin in egg white and yolk. The analyte was extracted from both parts of the egg and specifically detected at 370 nm. The detection was not compromised by degradation products of the analyte produced under stress conditions. Beer's range was found to be 2.50-37.50 µg/mL with correlation coefficient approaching 0.998. LOD and LOQ were found to be 0.15 and 0.47 µg/mL, respectively. The recovery, intraday accuracy and inter-day accuracy were found to be (98.49-100.89%), (99.12-101.78) and (98.13-104.53%), respectively. The method showed precision because relative standard deviation was less than 5%. The fortified and domestic eggs showed the presence of quercetin in both the parts. The results of the present study indicate that quercetin accumulates in egg, and the method is simple, accurate, repeatable and reproducible.

Keywords: Quercetin; Poultry feed; Flavonoids; Ultraviolet spectroscopy; Eggs.

INTRODUCTION

Quercetin, 2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxychromen-4-one, is a flavonoid, commonly distributed in fruits and plant-based foods (Lean *et al.,* 1999*;* Shi *et al.,* 2009). Being an antioxidant, it can chelate metal ions and scavenge oxygen free radicals (Boots *et al.,* 2008). Formica and Regelson (1995) have reported its usefulness in treating cardiovascular ailments such as atherosclerosis, hypercholesterolemia and others blood circulation problems. The literature also revealed its use in treating diabetes, cataracts, hay fever, peptic ulcer, schizophrenia, inflammation, asthma, gout, viral infections, chronic fatigue syndrome, chronic infections of the prostrate and cancer (Russo *et al.,* 2012; Xing *et al.,* 2001). Besides medicinal value, it is reported to improve athlete's performance (Davis *et al.,* 2009). Keeping such points in view, plant materials rich in flavonoids including quercetin are added in poultry feed to pass on their benefits in food.

The fortified/enriched-feed is used to enhance poultry performance; prolong shelf-life of meat and increase egg laying rate, shell strength, protein contents and decrease fats in yolk. Fortified eggs and domesticated-hen eggs are expensive than the normal layer eggs, and their use is increasing day-by-day. To enhance the nutritional value of the eggs, Taga *et al.* (1984) reported the use of plant-based feed containing flaxseed or Chia seeds (2.0×10^{-4} mg/Kg quercetin), rice bran, sea kelp and alfalfa. Such feed additives increase essential nutrients in poultry that are then transferred to eggs. The addition of quercetin in feed is reported to increase laying rate, egg shell strength, protein contents and Haugh Unit (measure of egg protein quality based on the height of its egg white), and decrease lipid content of the yolk (Liu *et al.,* 2013). Such feed increases shelf-life of meat by reducing the rate of lipid oxidation (Goliomytis *et al.,* 2014). Using such feed, quercetin is supposed to be accumulated in eggs, which has yet not been verified. To verify it, there is need of specific method to determine quercetin contents in eggs.

For the determination of quercetin in plant materials, a number of methods have been reported involving ultraviolet spectrophotometry (Dowd, 1959), HPLC (da Queija *et al.,* 2001), capillary electrophoresis (Sun *et al.,* 2001) and derivative spectrophotometry (Baranowska and Rarog, 2001). The method for its extraction from plasma and determination using HPLC coupled with electrochemical and spectrophotometric detection was described by Jones *et al.* (1998). However, the literature review has not indicated any report for the determination of quercetin in eggs. Therefore, the present study describes for the first time the determination of quercetin from egg white and yolk and its behavior under stress conditions.

MATERIALS AND METHODS

Solvents, chemicals and other supplies: The solvents of analytical grade procured from the local market included methanol, acetonitrile, formic acid and n-hexane (E-Merck, Germany). Analytical grade quercetin was procured from Sigma Aldrich, Germany. Different types of eggs were purchased from the market, while some eggs were obtained from our research collaborator of University of Veterinary and Animal Sciences, Lahore, Pakistan, who got the eggs after feeding flavonoids-enriched feed.

Instrumentation: The present work was carried out on UV/Vis spectrophotometer, Model No: 2550 Cat No: 206-24401-93, Serial No: A10844400603LP, 220V~ 50/60Hz 190VA (Shimadzu Corporation Kyoto, Japan), using 10 mm matched quartz cells.

Development and validation of method

Standard solution: A stock solution of quercetin (5.00 mg/mL) was prepared in methanol. Then a range of working standard solution having concentration 2.50-500.00 µg/mL was prepared by diluting the stock solution with methanol.

Specificity: The specificity of the assay was evaluated by the following two ways.

i. Comparison of UV spectra: A standard solution and samples extracted from egg white and yolk having equal concentration of analyte were scanned in UV range (400-200 nm) and the resulting spectra were compared to find the wavelength which could detect quercetin unequivocally and without any interference.
ii. Forced degradation: *a)- Acid-degradation (Acidic hydrolysis):* Three milliliters of standard solution (1.0 mg/mL) were mixed with 1.0 mL of 3N HCl in a 10.0 mL of volumetric flask and the volume was made up with methanol. Then, it was kept at room temperature. Aliquots (1.0 mL) withdrawn after 60 and 90 min were neutralized with 2N NaOH in a 10.0 mL volumetric flask and volume was made up with methanol. Then, absorbance was measured against a blank, prepared by mixing 0.5 mL each of 3N HCl and 3N NaCl and diluting it to 10.0 mL with methanol (Narasimha *et al.*, 2013).

b)- Alkaline degradation (Basic hydrolysis): The procedure described for acid hydrolysis was used for alkaline degradation using 0.1N NaOH. The samples were neutralized using 0.1N HCl and the blank solution was prepared with same procedure as prepared for acid hydrolysis.

c)- Dry heat induced-degradation: The analyte was kept in an oven at 70ºC and after 48 h an appropriate amount was dissolved in methanol and the absorbance was measured at 370 nm against methanol as a blank.

d)- Oxidative degradation: A standard solution (1.5 mL) having concentration (1.0 mg/mL) and 1.0 mL of 35 % hydrogen peroxide were mixed in 10 mL volumetric flask and the volume was made up with methanol. A blank was prepared by diluting 1.0 mL of 35% to 10.0 mL with methanol. The sample and blank were kept at room temperature for 15 min and then boiled on water bath to remove the excess of hydrogen peroxide. Both of the solutions were diluted appropriately and absorbance of the sample was measured at 370 nm against the blank.

Extraction of quercetin from egg white: One milliliter egg white was spiked with 500.0 µL of quercetin standard solution (equivalent to 250.0 µg quercetin) and mixed thoroughly for 30 sec by vortex (SLV-6, Korea). One milliliter of acetonitrile was added and mixed by vortex for 30 sec. Then 3.0 mL of methanol (acidified with 300.0 µL of formic acid) was added and mixed by vortex for 1 min. The mixture was centrifuged at 5000 rpm for 15 min at 20^0C (Centrifuge, Sigma 2-16KC, Germany). The supernatant was collected and the residue was again extracted with another 3.0 mL of methanol. The pooled supernatant was evaporated *in vacuo* at 40^0C (Rotary evaporator, Model No. Laborta 4002, Heidilph, Germany) and the residue was reconstituted with 1.0 mL methanol.

Extraction of quercetin from egg yolk: The extraction was carried out from egg yolk using the method similar to that of the egg white. The supernatant was partitioned with 5.0 mL of n-hexane to remove fatty material. The fat-free material was evaporated *in vacuo* at 40^0C, and the residue was reconstituted with 1.0 mL methanol.

Linearity (Beer's Law range): The egg white and yolk samples were spiked with a range of standard solutions of quercetin (2.5-37.5 µg/mL) and extracted as mentioned above. The extracted samples were analyzed in triplicate at 370 nm. The absorbance was plotted versus concentration and linearity was assessed visually and correlation of data points was found by the correlation coefficient (R^2).

Recovery: Three samples of egg white and yolk were spiked separately using standard solutions having concentration 5.0, 10.0 and 15.0 µg/mL. The spiked samples were treated as mentioned above for the extraction of the analyte. The un-spiked egg white and yolk were also extracted, similar to that of the spiked, to prepare blanks. The samples were analyzed in triplicate and amount of the analyte was determined from calibration curves. The recovery was calculated by comparing the determined value to that of the true value.

Intra- and inter-day accuracy and precision: The samples prepared from the spiked egg white and yolk with three standard solutions (5.0, 10.0 and 15.0 µg/mL) were extracted and analyzed six times in a single day for

intraday accuracy and precision. For inter-day accuracy and precision, these samples were analyzed once every day for six consecutive days. The amounts were determined from calibration curves that were constructed on each day.

Sensitivity: Limit of detection (LOD) and Limit of quantification (LOQ): LOD and LOQ were determined statistically by constructing five standard curves using five standard solutions prepared after extraction from the egg white and yolk. The mean slope and standard deviation of intercept of the standard curves were used to calculate LOD and LOQ.

Robustness: The robustness of the assay was assessed by making changes in λmax (± 2) and determining the recovery values.

Application of the method for eggs of different types: The egg white and yolk were separated and extracted as described above. The samples obtained were analyzed at 370 nm. Five standard solutions, prepared after extraction, were analyzed similar to that of the samples to construct calibration curves for the determination of quercetin.

RESULTS AND DISCUSSION

Development and validation of method:

Specificity: The specificity of the method was assessed by extracting the analyte from egg white and yolk. The extracted samples were analyzed in UV range (400-200 nm) and the scans were compared with the solution of pure analyte. The scans shown in Figure 1 indicated that the UV profile of the extracted samples were not similar to that of the pure analyte's solution. However, quercetin standard solution showed maximum absorbance at two wavelengths such as 265 and 370 nm. The extracted samples from egg white and egg yolk gave different response at 265 nm. Moreover, total profiles of the extracted samples from egg white and yolk from 200 to 300 nm were different from the standard. This was due to the extracted components of egg white and yolk. However, the profiles of the extracted samples from both parts of the egg, and the standard solution, from 300-400 nm (maximum absorbance at 370 nm), were similar, which indicated that the determination at this wavelength was expected to be specific. The absorbance profile of the quercetin in the present study was found to be consistent to that reported earlier (Zvezdanović *et al.,* 2012). It showed maximum absorbance at wavelength 370 nm due to cinnamoyl moiety attached to the structure. The literature review revealed that flavonols exhibited two major absorption bands, one ranging from 320-385 nm and other from 250-285 nm. The attachment of additional functional groups might shift absorption to 370 nm as in quercetin due to cinnamoyl moiety (Kumar and Pandey, 2013).

Quercetin was subjected to forced degradation using acidic, basic, dry heat and oxidation conditions. Acid and base hydrolysis was performed by exposing the analyte to 3N HCl and 2N NaOH, respectively, for 90 min. The sample was exposed to dry heat (70^0C) for 48 h and H_2O_2 for overnight. The results of degradation studies are presented in Table 1. These results indicated that the analyte was degraded significantly due to acidic and basic hydrolysis. The degradation was further found to be increasing with the passage of time. The analyte was found to be stable to some extent against stressful conditions of dry heat and oxidation. Under such stress conditions, the assay was found to be capable of determining the concentration of quercetin. Despite unprecedented popularity due to wide range of therapeutic uses, quercetin has not been investigated for stress studies. However, *Gingko biloba* extract containing quercetin, kaempferol and isorhamnetin was investigated for stress studies and studies were conducted for 7 days (Jin *et al.,* 2013). Contrary to this, the present study was conducted under highly stressful hydrolytic conditions.

Extraction of analyte from egg white and yolk: The present study was conducted for quantification of quercetin in eggs. Hence, liquid extraction approach was applied to extract the analyte from egg white and egg yolk. Quercetin has good protein binding (Boulton *et al.,* 1998), hence there is a need of a solvent which can weaken analyte-protein binding. So, acetonitrile was used that weakens hydrophobic interactions leading to denaturation (Gekko *et al.,* 1998). This acetonitrile-aided denaturation of egg proteins resulted in limiting the protein-quercetin binding. Then, various types of solvents such as ethanol, methanol, mixture of water and methanol, water and acetic acid were used to extract the analyte from the denatured egg white. Methanol proved to be relatively a good solvent for such purpose. But, the main problem encountered was the turbidity of the extracted solution. Nevertheless, the solvent system comprising methanol and formic acid in the ratio of 10:1 (v/v) extracted the analyte from the egg white efficiently without any turbidity.

The same procedure was adopted for the extraction of the analyte from egg yolk. Nevertheless, the extracted sample found to have some lipid contents, which were then removed by partitioning with n-hexane.

Egg is always recognized as a food containing high nutritional quality for human and due to the afore-mentioned reason its demand in the market is always increasing. Besides this, it contains many biologically active compounds as it is directly involved in the formation of a new individual (Akkouche *et al.,* 2012). Egg white is primarily composed of about 90% water and 10% proteins (including albumins, glycoproteins /

mucoproteins and globulins) which are just 50% of the proteins in the whole egg. On the other hand, egg yolk contains high amount of lipids and some amount of carbohydrates (< 1%) (Mine, 1995). Egg white contains almost no fat whilst egg yolk has a significant amount of lipids along with vitamins, minerals and pigments (Sugino *et al.*, 1996). Therefore, the sample prepared from egg yolk required the removal of lipids by partitioning.

Nowadays, enriched or fortified eggs have captured much attention of users due to their tremendous benefits. The active principles of the herbs are accumulated in the eggs (Singh *et al.*, 2012). Therefore, such eggs may contain quercetin, which is an important flavonoid found in many plant-based feed and known for antioxidant properties.

Linearity: The Beer's law was found to be obeyed in a concentration range (2.5-37.5 µg/mL). The calibration curve between concentration and absorbance after extracting the analyte from the egg white is shown in Figure 2. The linear regression equation was found to be Y = 0.029 X + 0.216, with correlation coefficient (R^2 = 0.997). On the other hand calibration curve of the extracted samples from egg yolk is shown in Figure 3. This curve gave the linear regression equation Y= 0.021 X + 0.11 with correlation coefficient (R^2 = 0.992). Both of the standard curves indicated that the intercept values were approaching to zero.

Recovery, intraday and inter-day accuracy and precision: The results of recovery, intraday and inter-day accuracy and precision of the method are shown in Table 2 (for egg white) and Table 3 (for egg yolk). These results indicated that the method had good recovery, intraday accuracy and precision and inter-day accuracy and precision. Therefore, the method was reliable, repeatable and reproducible, hence could be used to determine quercetin from eggs with confidence.

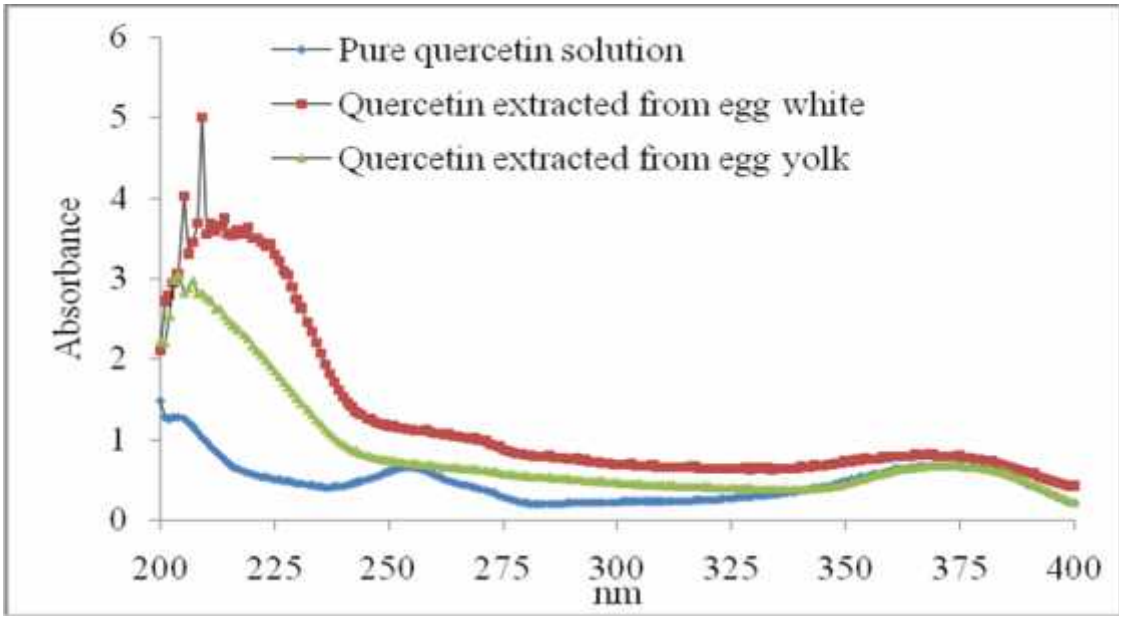

Figure 1 UV profiles of quercetin standard solution (40 µg/mL) and samples extracted from egg white and yolk both having 40 µg/mL quercetin.

Table 1. Stess conditions for degradation studies of quercetin.

Condition	Time	% Degradation
3N HCl	60 min	80.56
	90 min	89.2
0.1N NaOH	60 min	75.83
	90 min	94.31
35% Hydrogen peroxide	15 min	5.56
Dry Heat	48h	5.56

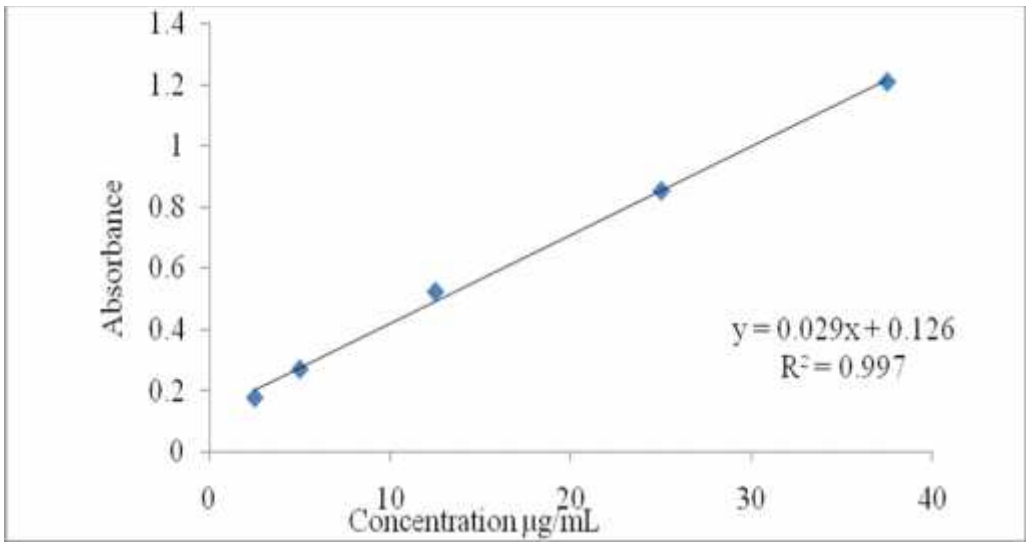

Figure 2. Calibration curve of quercetin extracted from egg white and detected at 370 nm.

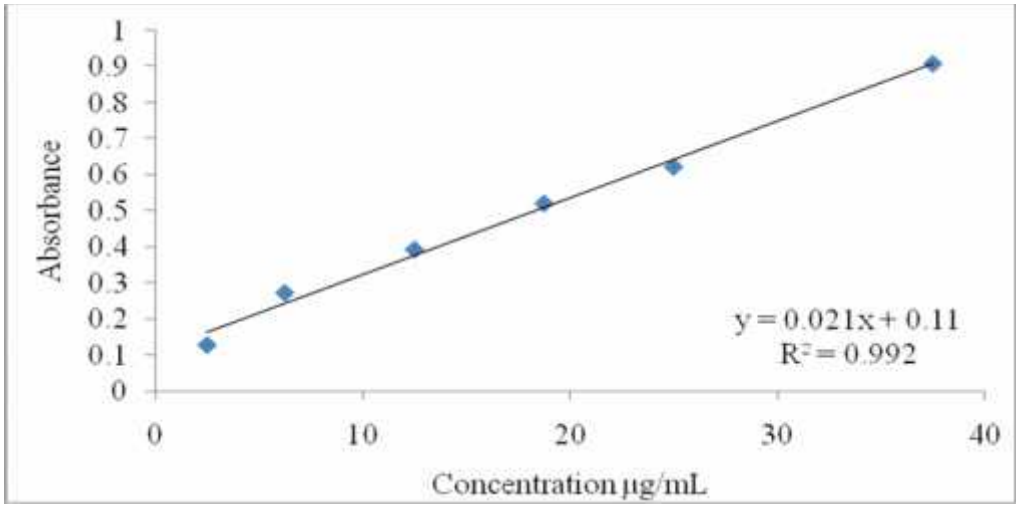

Figure 3. Calibration curve of quercetin extracted from egg yolk and detected at 370 nm.

Table 2. Recovery, intra and inter-day accuracy and precision values of quercetin extracted from egg white by UV spectrophotometry.

Concentration (µg/mL)	Recovery (n=3)		Intraday (n=6)		Inter-day (n=6)	
	Mean (%)	SD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
5.00	100.75	0.001	101.78	1.01	102.79	4.28
10.00	99.56	0.002	101.35	2.13	104.53	2.53
15.00	98.48	0.002	99.12	1.31	98.13	0.77

Table 3. Recovery, intra- and inter-day accuracy and precision values of quercetin extracted from egg yolk by UV spectrophotometry.

Concentration (µg/mL)	Recovery (n=3)		Intraday(n=6)		Inter-day(n=6)	
	Mean (%)	SD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
5.00	100.89	0.002	100.04	0.025	98.19	1.90
10.00	99.110	0.002	99.680	0.034	101.78	2.50
15.00	101.29	0.005	100.735	0.013	99.04	0.73

Sensitivity: LOD and LOQ: The values of LOD of the method from egg white and egg yolk were found to be 0.156 µg/mL and 0.153 µg/mL, respectively. The values of LOQ from egg white and egg yolk were found to be 0.472 µg/mL and 0.465 µg/mL, respectively. These results indicate that the method is quite sensitive.

Robustness: The samples were analyzed at 370 ± 2 nm and the recovery was found to be not effected by such changes. This indicated that the method was robust.

Application of the method for different types of eggs: This validated method was applied for the quantification of quercetin in egg albumen and egg yolk, obtained from different types of eggs. Egg albumen (white) was found to contain 8.85 and 8.78 µg/ml quercetin in domestic and fortified eggs, respectively. Egg yolk was found to contain 7.41 and 6.36 µg/ml quercetin in domestic and fortified eggs, respectively. To confirm these results, the analyte extracted from egg white and yolk of both types of eggs were scanned in UV range. The scan of the quercetin extracted from 100 µL albumen and yolk of both the types of eggs are given in Figure 4 and 5, respectively. The scans of quercetin extracted from egg albumen did not show any peak at 370 nm, which was seen in case quercetin. The same type of behavior was noted in case of quercetin extracted from egg yolk of both types of eggs. However, these scans were not at baseline and exhibited some absorbance in a region from 300-400 nm. The absence of peak at 370 nm need to be further verified using high performance liquid chromatography or any other separation technique.

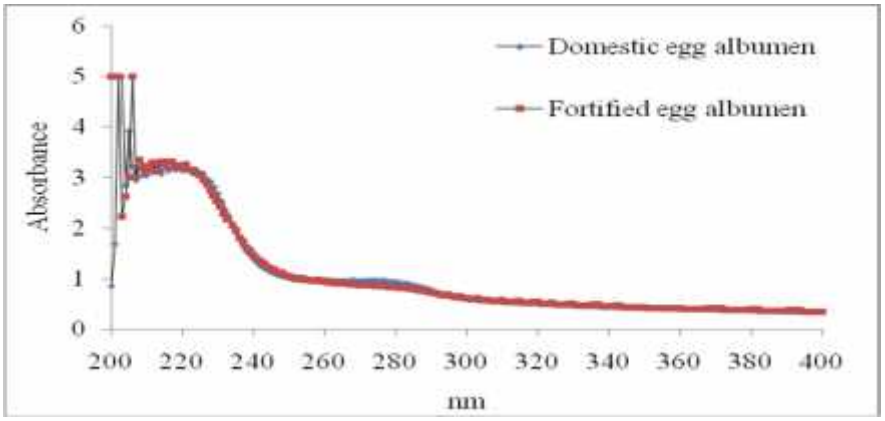

Figure 4. Overlays of UV spectra of samples extracted from 100 µL egg albumen of domestic and fortified eggs

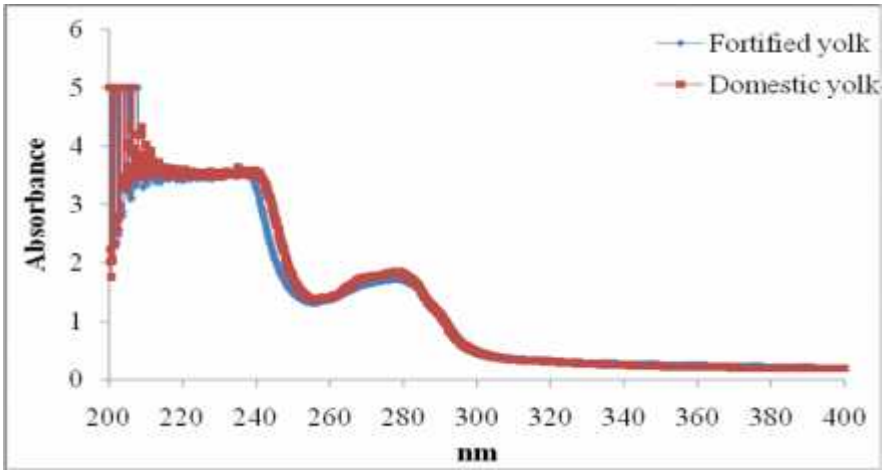

Figure 5. Overlays of UV spectra of samples extracted from 100 µL egg yolk of domestic and fortified eggs.

Conclusion: The present UV spectroscopic method is sensitive, specific, simple and appropriate to be used for the determination of quercetin in egg albumen and egg yolk. The eggs of domesticated hen and fortified eggs obtained from market contain quercetin in both albumen and yolk.

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