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Analytical Methods

Comparison of three spectrophotometric methods for analysis of egg yolk carotenoids

K.M.S. Islam [a,*], F.J. Schweigert [b]

[a] Department of Animal Nutrition, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[b] Institute of Nutritional Science, University of Potsdam, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany

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ABSTRACT

Carotenoids accumulated in the egg yolk are of importance for two reasons. Firstly they are important pigments influencing customer acceptance and secondly they are essential components with positive health effects either as antioxidants or as precursor of vitamin A. Different analytical methods are available to quantitatively identify carotenoids from egg yolk such as spectrophotometric methods described by AOAC (Association of Official Analytical Chemists) and HPLC (High Performance Liquid Chromatography). Both methods have in common that they are time consuming, need a laboratory environment and well trained technical operators. Recently, a rapid lab-independent spectrophotometric method (iCheck, BioAnalyt GmbH, Germany) has been introduced that claims to be less time consuming and easy to operate. The aim of the current study was therefore to compare the novel method with the two standard methods. Yolks of 80 eggs were analysed as aliquots by the three methods in parallel. While both spectrometric methods are only able measure total carotenoids as total ß-carotene, HPLC enables the determination of individual carotenoids such lutein, zeaxanthin, canthaxanthin, ß-carotene and β-apocarotenoic ester. In general, total carotenoids levels as obtained by AOAC were in average 27% higher than those obtained by HPLC. Carotenoid values obtained by the reference methods AOAC and HPLC are highly correlated with the iCheck method with r^2 of 0.99 and 0.94 for iCheck *vs.* AOAC and iCheck *vs.* HPLC, respectively (both $p < 0.001$). Bland Altman analysis showed that the novel iCheck method is comparable to the reference methods. In conclusion, the novel rapid and portable iCheck method is a valid and effective tool to determine total carotenoid of egg yolk under laboratory-independent conditions with little trained personal.

1. Introduction

Carotenoids are natural yellow to red pigments in plants that have different functions in man and animal. They are important pigments in food such as egg, function as antioxidants and as precursors of vitamin A (IARC, 1998; Schweigert, 1998; Surai & Sparks, 2001). Among the total number of 600 carotenoids the most common carotenoids are α-carotene, ß-carotene, ß-cryptoxanthin, lutein, zeaxanthin and lycopene. The carotenoid content in chicken feed and egg yolk can be analysed by different chemical analytical methods especially for the purpose of controlling the pigmentation grade of egg yolks primarily for customer acceptance (Hudon, 1994). The simplest one is the spectroscopic determination of total carotenoids as equivalents of ß-carotene (AOAC, 1958, 1973). Using High Performance Liquid Chromatography (HPLC) carotenoids can not only be quantified, but individual carotenoids can be separated (Hamilton, 1992; Schlatterer & Breithaupt, 2006; Steinberg, Grashorn, Klunter, & Schierle, 2000). Both methods have in common that they are dependent on a complex laboratory environment and sophisticated and expensive equipment as well as trained technical personal, especially in the case of HPLC.

An alternative method that is laboratory independent and requires very limited technical experience has recently been introduced namely iCheck (iCheck CAROTENE, BioAnalyt GmbH, Germany). This test system consists of two components, a portable easy to handle LED spectrophotometer and a disposable all-in-one analytical unit to extract and quantify carotenoids in one step. Such an easy to use method is convenient especially in low-resource settings of developing countries.

Aim of this study is to compare the results produced by the new method with results obtained by the two standard methods such as the AOAC method using a spectrophotometer and the HPLC method.

* Corresponding author. Tel.: +880 91 67401 to 6x2602, mobile: +880 1715226510; fax: +880 0088 091 61510.
E-mail address: kmsislam1@yahoo.com (K.M.S. Islam).

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2. Materials and methods

2.1. Chemicals

All chemical products were analytical grade. Acetone was obtained from Merck (Darmstadt, Germany); other chemicals were collected from BioSolve (Sankt Augustin, Germany).

2.2. Eggs

Eggs were obtained from 8 difference sources (6 chickens, 1 duck, 1 quail) at local markets, a commercial farmer, small private producers and the zoological garden of Berlin, Germany. A total number of 80 eggs (10 from each source) were considered. Eggs were cleaned by distilled water and dried at room temperature. After breaking the eggs albumen and egg yolk was separated. Yolk content was collected after rupturing the yolk membrane and the yolk was thoroughly mixed and aliquoted before analysis. Each yolk was extracted and analysed in triplicates for each method.

2.3. Spectroscopy AOAC method

According to AOAC method (AOAC, 1958, 1973) one gram of well mixed egg yolk from each egg was taken in a conical flask where acetone was added in two steps, first 1 ml to make a smooth past and thereafter 50 ml. The solution was well mixed and filtered (equivalent to Whatman Nr. 4). After washing the filter with acetone, the recovered acetone was diluted to 100 ml. Yolk pigmentation equivalent to µg ß-carotene/g sample was measured on a spectrophotometer at 450 nm wavelength (E1% 2500).

2.4. HPLC method

Briefly 1.5 g egg yolk was mixed with 4 ml of distilled water for 30 min using vortex and extracted two times with 5 ml solvent (*n*-hexane/isopropanol, 3:2 v/v) for 15 min on shaker. The sample was than centrifuged and the whole solvent collected in tube A after each time of extraction, this combined extraction solvent were washed by adding 5 ml 0.1 M NaCl, mixing vigorously, and incubating for 30 min until two layers were separated. The upper hexane layer was transferred to tube B. The remaining part of lower layer was vigorously washed with 7.5 and once more with 5 ml of *n*-hexane/BHT (0.05%) in each case for 30 min in darkness until two layers were separated. The upper hexane layers were removed to the tube B and the volume was filled up to 20 ml with *n*-hexane/BHT 0.05%. For HPLC analysis, 100 µl of this sample was evaporated to dry (Techne sample concentrator, model FDB03OD, Camlab Ltd, Cambridge, UK) and then re-dissolved in 200 µl isopropanol for HPLC injection.

The HPLC system (Waters GmbH, Eschborn, Germany) equipped with a binary pump system, a degasser, an auto-sampler and a diode array detector (DAD). The separation was carried out on a C30 column, 250 × 3 mm, 5 µm (YMC Europe GmbH, Dinslaken, Germany). The column temperature was kept at 20 °C. The binary mobile phase consisted of methanol–ammonium acetate, 0.4 g/l in distilled water (9:1, v/v; solvent A) and methyl-t-butyl ether–methanol–ammonium acetate, 0.1 g/l in distilled water (90:8:2, v/v/v, solvent B). The flow rate was kept at 0.2 ml/min. The gradient was: start with 5% B, in 1 min to 7% B, in 1 min to 15% B, in 1 min to 20% B, in 8 min to 25% B, in 10 min to 55% B, in 8 min to 87% B, in 3 min to 93% B, in 1 min to 99% B and 99% B for 14 min. For equilibration 13 min 5% B. Gradient was finished after 60 min. Detection was conducted at a wavelength of 450 nm. The HPLC systems were able to separate all E and Z isomers of β-apocarotenoic ester, canthaxanthin, lutein, zeaxanthin and ß-carotene, those were calculated based on calibration with reference substances (DSM Nutritional Products, Basel, Switzerland) of the all E carotenoid and using experimentally determined relative response factors for the Z isomers. For the determination of the total carotenoid content detected with non-identified carotenoids were quantified with the response factor of the dominant carotenoid in the chromatogram and summed up with the identified carotenoids like β-apocarotenoic ester (E1% 2640 in light petroleum at 457 nm), canthaxanthin (E1% 2200 in light petroleum at 466 nm), lutein (E1% 2550 in ethanol at 445 nm), zeaxanthin (E1% 2540 in ethanol at 450 nm) and ß-carotene (E1% 2540 in ethanol at 450 nm).

2.5. New method

The iEx/iCheck® method consists of a disposable all-inclusive extraction (iEx) and measuring unit, a battery-driven, hand-held photometer, the iCheck(™) (BioAnalyt GmbH, Teltow, Germany; www.bioanalyt.com). An amount of 400 mg of egg yolk was diluted to a final weight of 2.00 g with dilution buffer (8 M urea) using an incorporated fine balance. The volume of 400 µl of the diluted egg yolk was injected into the extraction vial with a disposable syringe. Thereafter, the vial was shaken intensively for 10 s and left for complete phase separation for at least 5 min. In that step carotenoids were completely separated into the upper organic phase. The concentration of total carotenoids was measured in the portable LED photometer at 450 nm. The final concentration (mg/kg) was calculated based on sample weight and final buffer weight as total ß-carotene.

2.6. Statistical analysis

Data were organised using computer Excel program and mean values from different sources of egg yolk were compared and one way ANOVA done by Duncan's Multiple Range Test (Steel & Torrie, 1990). Regression and correlation coefficients were drawn between the data obtained by iCheck and AOAC as well as iCheck and HPLC. Tests of agreement between the new method and the two reference method were performed using Bland–Altman test (Altman & Bland, 1983).

3. Results and discussion

3.1. Comparison of total carotenoid content determined by different methods

Total carotenoids concentration in egg yolks from different sources varied considerably independently of the analytical method used (Table 1). Such substantial variations are well described and due to variation of feed, breed, species and other factors (Bortolotti, Negro, Surai, & Prieto, 2003; Fletcher, Janky, Christmas, Arafa, & Harmas, 1977). A similar high variability was observed when individual carotenoids were analysed by HPLC (Table 2). Again such differences are highly dependent on the composition of the feed (Okonkwo, 2009).

Comparing the three methods one has to consider that the AOAC and the new iCheck CAROTENE method are very similar with regard to the quantification of the carotenoids. Both use the absorption at 450 nm and the extinction coefficient (E1%) of ß-carotene for the quantification as total carotenoids. But they are different with regard to the extraction procedure. However, the HPLC and the iCheck CAROTENE method are similar with regard to the extraction procedure and solvent composition. Both extract all carotenoids into hexane as organic solvent. Quantification was done at the same wavelengths but concentration was

Table 1
Total carotenoid concentration of egg yolk ($x \pm SD$) determined by iCheck, AOAC and HPLC method and comparison of iCheck with other methods.

Rearing (species)	Floor (chicken)	Floor (chicken)	Free (chicken)	Free (chicken)	Free (chicken)	Free (chicken)	Free (duck)	Free (quail)
% iCheck	$28.7^{bc} \pm 3.5$*	$22.5^{ab} \pm 5.8$	$28.3^{bc} \pm 6.6$	$22.9^{ab} \pm 5.1$	$25.6^{ab} \pm 19.8$	$38.1^{c} \pm 14.9$	$27.8^{bc} \pm 3.7$	$14.3^{a} \pm 6.8$
AOAC	$30.6^{bc} \pm 3.7$	$24.4^{ab} \pm 5.4$	$30.1^{bc} \pm 6.3$	$20.4^{ab} \pm 3.4$	$27.4^{ab} \pm 19.7$	$40.6^{c} \pm 15.2$	$29.9^{bc} \pm 5.1$	$15.6^{a} \pm 7.9$
HPLC	$21.7^{bc} \pm 2.4$	$18.7^{ab} \pm 4.0$	$22.9^{bc} \pm 5.0$	$15.7^{ab} \pm 3.3$	$16.9^{ab} \pm 12.4$	$30.2^{c} \pm 11.4$	$21.4^{bc} \pm 3.0$	$12.6^{a} \pm 4.8$
Comparison of values (%) among different methods								
% iCheck of AOAC	$94^{a} \pm 4$	$91^{a} \pm 4$	$94^{a} \pm 4$	$93^{a} \pm 10$	$91^{a} \pm 5$	$94^{a} \pm 9$	$94^{a} \pm 10$	$94^{a} \pm 22$
% iCheck of HPLC	$132^{bc} \pm 7$	$119^{ab} \pm 11$	$123^{ab} \pm 7$	$123^{ab} \pm 9$	$147^{c} \pm 14$	$126^{ab} \pm 7$	$130^{bc} \pm 10$	$114^{a} \pm 19$
% AOAC of HPLC	$14^{ab} \pm 9$	$130^{a} \pm 7$	$130^{a} \pm 9$	$132^{a} \pm 18$	$161^{b} \pm 15$	$136^{ab} \pm 19$	$147^{ab} \pm 19$	$127^{a} \pm 31$
Pearson correlation coefficient (r^2) with values from iEx/iCheck and other methods								
AOAC	0.95	0.99	0.99	0.92	1.00	0.98	0.82	0.90
HPLC	0.91	0.93	0.97	0.94	1.00	0.99	0.85	0.97

[a,b,c]Values in rows with different superscripts differ significantly ($p < 0.05$); ($n = 80$).
iCheck – BioAnalyt GmbH, Teltow, Germany; AOAC – Association of Official Analytical Chemists; HPLC – High Performance Liquid Chromatography.
* Standard deviation.

Table 2
Carotenoid components of the egg yolk (mg/kg yolk, $x \pm SD$) collected from different sources of Berlin, Germany.

Rearing (species)	Floor (chicken)	Floor (chicken)	Free (chicken)	Free (chicken)	Free (chicken)	Free (chicken)	Free (duck)	Free (quail)
Lutein	$7.09^{ab} \pm 0.77$*	$8.19^{ab} \pm 2.21$	$4.06^{a} \pm 1.23$	$3.42^{a} \pm 0.60$	$9.94^{b} \pm 7.97$	$17.74^{c} \pm 7.21$	$6.98^{ab} \pm 0.76$	$4.86^{a} \pm 2.06$
Zeaxanthin	$1.02^{ab} \pm 0.13$	$0.88^{a} \pm 0.16$	$1.03^{ab} \pm 0.38$	$0.70^{a} \pm 0.21$	$1.41^{ab} \pm 1.37$	$1.46^{ab} \pm 0.70$	$1.31^{ab} \pm 0.22$	$1.85^{b} \pm 0.89$
Canthaxanthin	$6.92^{c} \pm 1.53$	$4.48^{b} \pm 1.35$	$4.55^{b} \pm 1.37$	$5.24^{b} \pm 1.64$	0.0	0.0	$1.48^{a} \pm 0.22$	0.0
β-Apocarotenoic ester	0.0	0.0	$9.34^{c} \pm 2.89$	$3.29^{b} \pm 1.52$	$0.17^{a} \pm 0.08$	$0.52^{a} \pm 0.47$	$5.09^{b} \pm 1.41$	$0.34^{a} \pm 0.20$
ß-Carotene	$0.19^{a} \pm 0.02$	$0.19^{a} \pm 0.03$	$0.17^{a} \pm 0.03$	$0.13^{a} \pm 0.02$	$0.57^{b} \pm 0.65$	$1.15^{c} \pm 0.41$	$1.74^{d} \pm 0.16$	$0.12^{a} \pm 0.02$
Total (sum of above)	$15.22^{bcd} \pm 2.07$	$13.74^{bc} \pm 3.66$	$19.14^{cd} \pm 4.66$	$12.78^{abc} \pm 3.24$	$11.96^{ab} \pm 9.95$	$20.53^{d} \pm 8.42$	$16.60^{bcd} \pm 1.20$	$7.14^{a} \pm 3.10$

[a,b,c]Values in rows with different superscripts differ significantly ($p < 0.05$); ($n = 80$).
* Standard deviation.

calculated using the HPLC method as individual carotenoids based on their individual extinction coefficients.

Greatest quantitative differences were observed between the AOAC method and the HPLC method, the later one being in average 27% lower. The new methods studied was in closer agreement with the AOAC methods (lower by 7% on average) and still in average 22% higher than the HPLC method. Thus, for both spectrophotometric methods the greatest differences were observed with the HPLC method indicating that the major reason lies in the quantification difference rather than in differences with regard to extraction because the amount of sample took as per recommended for complete extraction (Schweigert, Schierle, & Hurtinne, 2010).

It has also been reported that spectrophotometric methods tend to overestimate carotenoid content when compared to HPLC due to other compounds also detected, for example, carotenoid degradation products (Kimura, Cobori, Rodriguez-Amaya, & Nestel, 2007) and chlorophyll degradation products such as chlorophyllides which are also absorbing at similar wavelengths (Almela, Férnandez-Lopéz, & Roca, 2000) despite the fact that their increased polarity due to phytol cleavage would result only in small extractability in hexane (Chiba et al., 1967). Lower carotenoid concentrations in HPLC methods may also be explained by saponification losses due to the relatively harsh conditions of alkaline treatment (Biehler, Mayer, Hoffmann, Krause, & Bohn, 2010; Khachik, Beecher, & Whittaker, 1986; Oliver, Palou, & Pons, 1998; Rodriguez-Amaya & Kimura, 2004). Especially the polar carotenoids are sensitive to such conditions (Schweigert, Hurtienne, & Bathe, 2000). Although similar types of work have not been conducted in egg yolk it can be concluded for our results that the observed underestimation in the HPLC method might be due to one or the other mentioned reason. Finally, some unidentified carotenoids present in the egg yolk are missed in the calculation because of unknown chemical structure (no external standards available) or concentrations at detection level. In our study we estimated that this can account for up to 10% of the difference. In another experiment with egg yolk the results of the iCheck method were in average 12% higher compared to results obtained by HPLC (Schweigert et al., 2010).

According to Pearson's correlation analysis (Table 1) values from the iCheck CAROTENE method have a close relationship with the values obtained by AOAC methods with an average of r^2 of 0.99 ranging from 0.82 (free range duck) to 1.00 (free range chicken). The relationship between the values obtained by the iCheck and HPLC method was in average r^2 of 0.94 ranging from 0.85 (free range duck) to 1.00 (free range chicken) (Figs. 1 and 2). With such high average r^2 value the agreement can be interpreted as almost perfect. Raila, Enjalbert, Mothes, Hurtienne, and Schweigert (2012) also demonstrated that both the iCheck and HPLC method showed a very good agreement based on correlation coefficient of $r^2 > 0.98$; ($p < 0.001$). In this case ß-carotene was determined in cattle blood plasma and results were compared to HPLC analysis. Results of this study support the good agreement between the new method and standard methods such as spectroscopy or HPLC.

3.2. Bland Altman analysis of different methods

Different statistical methods are available to test the inter changeability of methods and if a new method produces similar results then the established standard method. In this case the Bland–Altman plot analysis confirms that no systematic error exists between the new method and the two established reference methods (Fig. 3, Fig. 4). But, the data distributed in different pattern because data shows from AOAC method nearest and HPLC method lower in comparison to iCheck method. When data analysed to compare AOAC and HPLC method (Fig. 5) similar pattern of distribution observed as comparison between iCheck and HPLC method (Fig. 4), because data from AOAC method is nearest to the iCheck method.

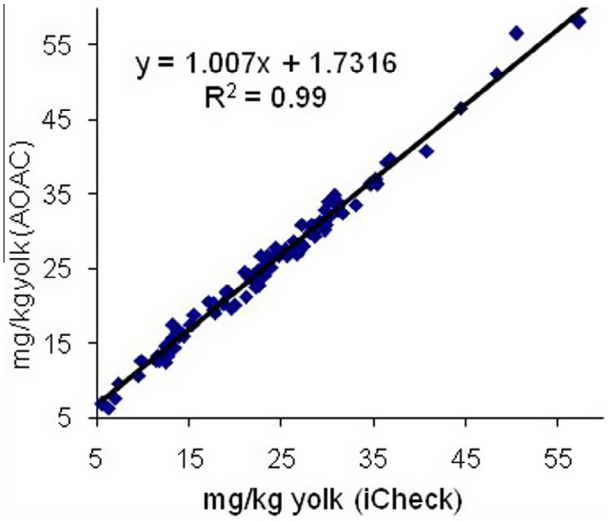

Fig. 1. Correlation between the values of total carotenoid (mg/kg yolk) measured by iCheck and AOAC method.

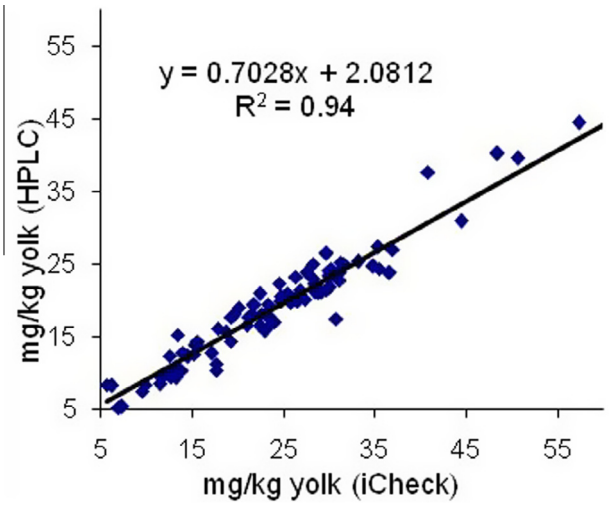

Fig. 2. Correlation between the values of total carotenoid (mg/kg yolk) measured by iCheck and HPLC method.

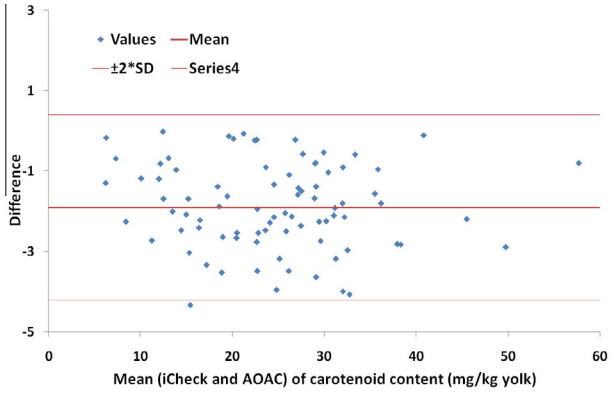

Fig. 3. Bland–Altman plot showing the mean difference between ß-carotene concentrations of egg yolk measured by AOAC and iCheck method.

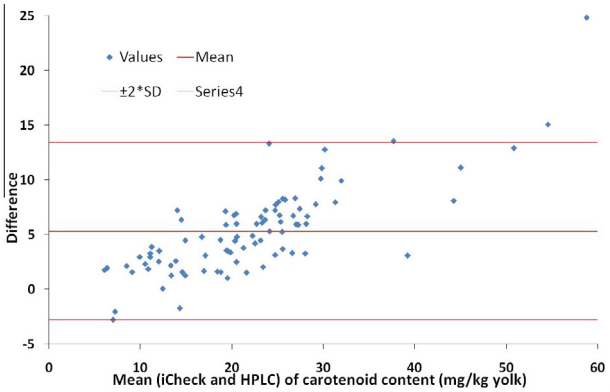

Fig. 4. Bland–Altman plot showing the mean difference between ß-carotene concentrations of egg yolk measured by HPLC and iCheck method.

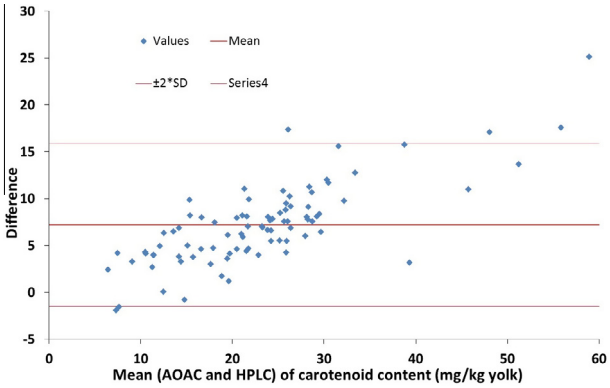

Fig. 5. Bland–Altman plot showing the mean difference between ß-carotene concentrations of egg yolk measured by AOAC and HPLC method.

4. Conclusions

In conclusion the comparison of the novel iEx/iCheck method for total carotenoids in egg yolk showed a very good agreement between the new method and the two standard methods AOAC and HPLC. In comparison to the sophisticated, but time consuming, laboratory based and expensive AOAC and HPLC methods the new method is able to analysis egg yolk within few minutes. The low technical prerequisites and its portability makes the method very suitable to be used even in a poultry shed. In addition to practical considerations and reduced cost of investment the new test system also reduces the exposure of the laboratory personal to potentially dangerous organic solvents and uses much less organic solvents compared to the conventional methods thus reducing the environmentally and ecologically critical waste to a minimum. All these aspects taken together classify the method as highly suitable for low resource settings especially in developing countries.

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