Analysis of Total Carotenoids in Egg Yolk - A Fast and Laboratory-Independent Assay

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Summary

Carotenoids are essential ingredients in animal feed. They are not only important as pigments but serve as health promoting antioxidants and as precursor of vitamin A. Because of their importance in visual perception of animal products such as eggs and their central role in health and well being of the animal, carotenoid content needs to be carefully monitored. Current analytical techniques to measure total carotenoids in egg or egg products are time-consuming and equipment-intensive. We compared an innovative new on-step method with a hand held photometer (iEx, iCheck, BioAnalyt GmbH Germany) to determine total carotenoids from egg yolk, with the AOAC method and with HPLC as reference methods. Results of this comparison of both methods show a very good agreement based on correlation coefficients of > 0.96 and examination of the Bland-Altman differences blot reveal a good agreement with no real bias. Differences of the new method with AOAC were below 5 %. The 14 % higher values reported for HPLC are acceptable considering that HPLC results are derived from a different quantification principal. Comparing the necessary time and technical equipment, the innovative one-step extraction system is superior to the traditional laboratory methods with regard to the ease of application and gives a very good agreement to the standard methods. In conclusion, for the first time a quantitative analysis of total carotenoids can be performed in egg and egg products at production site with very little equipment and within minutes. This will greatly enhance the ease of application and improve the product quality.

Key words: carotenoids, egg yolk, analysis, method, HPLC, spectroscopy

Introduction

Carotenoids are essential ingredients in animal feed . They are of central importance as pigments but serve as health promoting antioxidants and as precursor of vitamin A (Schweigert, 1998, Surai and Sparks, 2001). The colour of a yolk can only be predicted and controlled through the control of the carotenoid content in the feed (Hudon, 1994). Controlling the colour of the egg yolk matters a great part because consumers in various cultures prefer yolks of certain defined and reproducible hues of yellow-orange colours (Bender, 1981).

The simplest method is the estimation of carotenoids with the eye. The DSM Yolk Colour Fan (DSM-YCF) has become the instrument most commonly used to measure the colour of the egg. This method however only gives information of colour but not on the content of the biologically important carotenoids. Therefore, different chemical methods have been developed to quantitate either total carotenoids or individual carotenoids in egg. The simplest one is the spectroscopic determination of total carotenoids as equivalents of ß-carotene (AOAC, 1958, 1973). To determine the concentration of individual carotenoids, however, the carotenoids have to be separated by HPLC prior to analysis (Schlatterer and Breithaupt, 2006) (Hamilton, 1992). Both methods need a laboratory environment and different degrees of sophisticated technical equipment.
In this paper a new, fast, easy to perform and laboratory-independent assay for the determination of total carotenoids in egg and egg products is described. The test consists of two components, a portable easy to handle spectrophotometer and a disposable all in one analytical unit. The results produced by the new analytical method are compared with those obtained by the AOAC method (AOAC, 1958, 1973) and by HPLC (Steinberg *et al.*, 2000) used as reference methods.

Material and Methods

Eggs were bought at random at the local market. Albumen and egg yolk were separated, and the egg yolk was thoroughly mixed to be separated into equal amounts for comparative analysis between the new method and the AOAC method. For the comparison with HPLC, laying hens were fed in the animal nutrition research centre of DSM Nutritional Products, CRNA, NRD/CA in Village Neuf, France, with diets containing various concentrations and combinations of apoester (ethyl-β-apo-8'-carotenoate as CAROPHYLL® Yellow 10%), canthaxanthin (as CAROPHYLL® Red 10%) and Marigold carotenoids lutein and zeaxanthin. For each treatment 4 repetitions with 12 hens each were run. Four yolks from each repetition were pooled.

New method: The iEx/iCheck® method consists of a disposable all including extraction and measuring unit, the iEx(TM) and a battery driven hand-held photometer, the iCheck(TM) (BioAnalyt GmbH, Teltow, Germany). 400 mg of egg yolk are diluted to a final weight of 2.00 g with dilution buffer using an included fine balance. 400 µl of the diluted egg yolk are injected into the extraction vial. Thereafter, it is shaken for 10 seconds vigorously and left for complete phase separation for at least 5 minutes. This completely extracts all carotenoids present in the sample into the upper organic phase. The concentration is measured in the portable photometer and final concentration (mg/kg) is calculated based on sample weight and final buffer weight.

Spectroscopy: The AOAC method (AOAC, 1958, 1973) was performed with the following modifications. To 1 g of egg yolk from one egg acetone was added in two steps, first 1 ml to make a smooth past and thereafter 50 ml. The solution was mixed and filtered (equivalent to Whatman Nr. 4). After washing the filter with acetone, the recovered acetone was diluted to 100 ml. Yolk colour equivalent to µg ß-carotene/g sample was measured on a spectrophotometer at 450 nm wavelength.

HPLC: Analyses of the egg yolk material were conducted as described elsewhere (Steinberg *et al.*, 2000). The egg yolk materials were mixed with water and ethanol and the mixtures liquid-liquid extracted with n-hexane. Aliquots of the upper hexane phase were injected into various isocratic normal-phase HPLC systems which used all the same stationary phase

(LiChrosorb Si60, 250x4 mm) but different acetone/n-hexane mixtures as mobile phases (0.5 – 19% acetone, depending on the target carotenoid). The HPLC systems were able to separate and quantify all-E- and Z-isomers of apoester, canthaxanthin, lutein, and zeaxanthin. The calculation of the contents was based on calibration with reference substances of the all-E-carotenoids and using experimentally determined relative response factors for the Z-isomers. For the determination of the total carotenoid content detected but non-identified carotenoids were quantified with the response factor of the predominant carotenoid in the chromatogram and summed up with the identified carotenoids.

Statistical Analysis: The results were analysed by three different methods. Correlation of results obtained were analysed using regression analysis. The degree of agreement between the two methods was evaluated by examination of the Bland-Altman difference plots. Good agreement with no real bias was indicated when the 95 per cent confidence interval for the bias including zero (Bland and Altman, 1986).

Results and Discussion

Comparison with the AOAC method: Table 1 shows the results for five different groups of egg yolks with five samples each. These five samples were analysed as triplicates. Results indicate that there is now significance difference for all egg yolk samples between the two methods. The levels in the AOAC group were slightly higher. As shown in Fig. 1 correlation between the two methods were excellent for total carotenoids. With r values above 0.98 the agreement can be interpreted as almost perfect. Examination of the Bland-Altman differences plots revealed for the individual groups that both methods were in good agreement, with no real bias for measurement of total carotenoids (Fig. 2). Based on five determinations of each sample the intra-individual coefficient of variation (CV) was calculated for each method. In average the CV was in an order of magnitude generally accepted for average analysis of this kind. With 2.3 vs.3.5 % CV was higher in the AOAC method compared to the new method.

Comparison with HPLC: Table 2 shows a comparison of the carotenoid contents measured by HPLC and with the new iCheck assay in egg yolks containing increasing amounts of the yellow carotenoid apoester besides traces of lutein and zeaxanthin. It is obvious from the results that iCheck found higher total carotenoid contents compared to HPLC and that this overestimation depends on the amount of the extracted sample portion. Low apoester concentrations of up to 10 ppm were clearly overestimated by the iCheck assay if sample portions of 0.4 g were extracted. This overestimation could be reduced to <10% when portions of 0.8 g yolk were extracted indicating that the measurement by iCheck was out of the linear range at very low carotenoid concentrations. Apoester concentrations in the yolk of approx. 15 to 300 ppm were overestimated by iCheck by less than 10% when sample portions of 0.4 g were used for yolks with less than 100 ppm total carotenoid and sample portions of 0.2 g for yolks with more than 100 ppm total carotenoid. Extraction of the highest concentrated egg yolk (approx. 270 ppm apoester) gave a similar result independently if sample portions of 0.2 or 0.1 g were used indicating that a sample portion of 0.2 g is suited also for the highest carotenoid concentrations.

If the yolk contained mainly canthaxanthin besides minor amounts of the yellow carotenoids lutein and zeaxanthin, the iCheck assay led to lower total carotenoid contents compared to HPLC (Table 3). This underestimation was expected since canthaxanthin is a reddish pigment with an absorption maximum at approx. 466 nm which is not adequately covered by the β-carotene equivalent method using a detection wavelength of 450 nm. At very low total

carotenoid concentrations (of <10 ppm) the underestimation was more pronounced for sample portions of 0.8 g than for 0.4 g suggesting that the underestimating effect due to the non-ideal wavelength was countered by an overestimation due to the limited linearity of the assay at such low concentrations. This finding is certainly more of academic interest than of practical importance since canthaxanthin and other red carotenoids are usually fed in combination with yellow carotenoids in order to obtain an acceptable yolk colour.

In yolks with various combinations of canthaxanthin and apoester the iCheck analyses of the egg yolks agreed within 12 % with the HPLC results (Table 3). In accordance with the finding above, carotenoid mixtures with higher canthaxanthin concentrations and lower contribution of yellow carotenoid tended to be underestimated whereas those with higher apoester concentrations were rather overestimated by the iCheck assay.

Table 4 shows results obtained for egg yolks containing mainly the yellow xanthophylls lutein and zeaxanthin in concentrations varying from approx. 10 to 100 ppm. In these cases the iCheck assay found up to 15% lower total carotenoid contents. For egg yolks with concentrations higher than 10 ppm there was no significant effect of the sample portion varying between 0.4 and 0.2 g. At lower concentrations, the extraction of 0.8 g egg yolk led to a higher underestimation (by 15%) compared to a sample portion of 0.4 g. This may indicate that at low concentrations in the measuring solution the underestimation was countered by a linearity effect.

Conclusion: The comparison of the new iCheck assay to analyse total carotenoids in egg yolk showed an excellent agreement with the standard AOAC method. This is expected as both methods use the total absorption of all present egg yolk carotenoids and are based on the calibration with ß-carotene as an external standard. However, the iCheck results also agreed well (i.e. within 15%) with the total carotenoid contents measured by HPLC in a wide range of carotenoid concentrations and combinations. This good correspondence is remarkable as the HPLC results derive from a different quantitation principle representing the sum of separated and specifically quantified carotenoids. Comparing the necessary time and technical equipment, the innovative one-step extraction system is superior to the traditional methods with regard to the ease of application and gives results comparable to the standard methods. The analytical quality is in excellent agreement to the laboratory analyses by the standard AOAC method and in acceptable accordance to HPLC. Besides its easiness of performance and analytical sensitivity, the novel system is ecologically superior to prior methods. The miniaturized all-in-one system reduces the exposition of the laboratory personal to potentially dangerous organic solvents to a minimum thus reducing the environmentally and ecologically critical waste dramatically.

References

AOAC (1958) AOAC final action method 17.002, color of eggs. *Journal of the Association of Official Analytical Chemists* **41**: 274.

AOAC (1973) AOAC final action method 17.002, color of eggs. *Journal of the Association of Official Analytical Chemists* **56**: 272.

BENDER, A.E. (1981) The appearance and the nutritional value of food products. J*ournal of Human Nutrition* 35: 215-217.

BLAND, J.M. and ALTMAN, D.G. (1986) Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **1**: 307-310.

HAMILTON, P.B. (1992) The use of high-performance liquid chromatography for studying pigmentation. *Poultry Science* **71**: 718-724.

HUDON, J. (1994) Biotechnological applications of research on animal pigmentation. *Biotechnology Advances* **12**: 49-69.

SCHLATTERER, J. and BREITHAUPT, D.E. (2006) Xanthophylls in commercial egg yolks: quantification and identification by HPLC and LC-(APCI)MS using a C30 phase. Journal of *Agricultural Food Chemistry* **54**: 2267-2273.

SCHWEIGERT, F.J. (1998) Carotenoids metabolism in mammals, in: BRITTON G, LIAAN-JENSEN S and PFANDER H (Eds). *Carotenoids*, pp. 249-284 (Basel, Boston, Berlin, Birkhäuser Verlag).

STEINBERG, W., GRASHORN, M., KLUNTER, A., and SCHIERLE, J. (2000) Comparative pigmentation efficiency of two products containing either apo-ester or tagetes extracts in egg yolks and liquid eggs. *Archiv für Geflügelkunde* **64**: 180-187.

SURAI, P.F. and SPARKS, N.H.C. (2001) Designer eggs: from improvement of egg composition to functional food. *Trends in Food Science and Technology* **12**: 7-16.

Figure 1: Correlation of the total carotenoid results with the new method and the standard AOAC method.

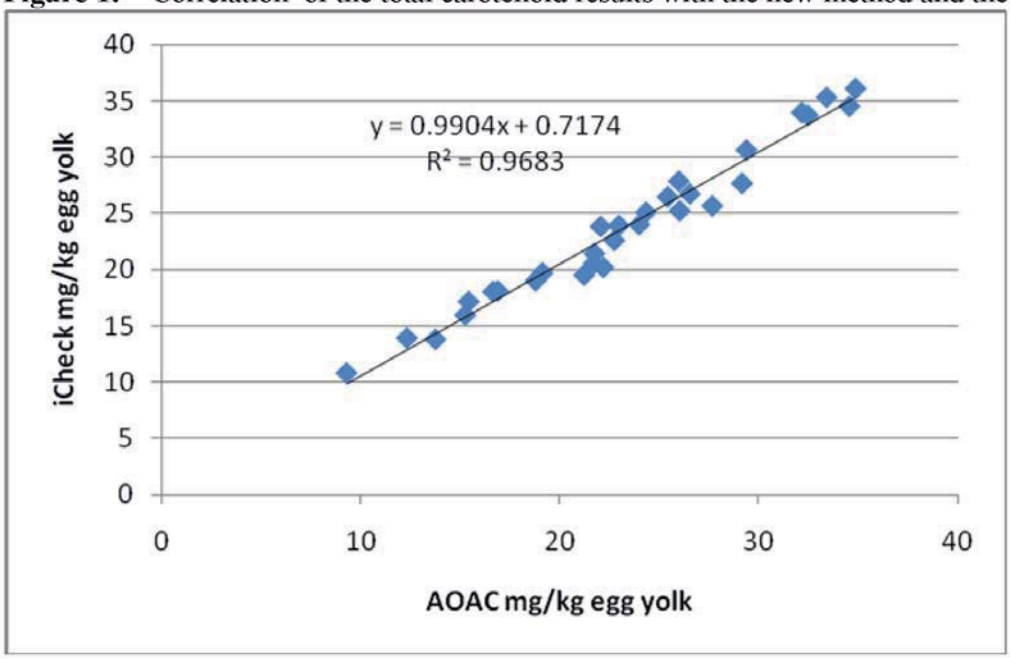

Figure 2: Bland-Altman blot of the total carotenoid levels from egg yolk determined with the new method (iCheck) compared with the standard AOAC method

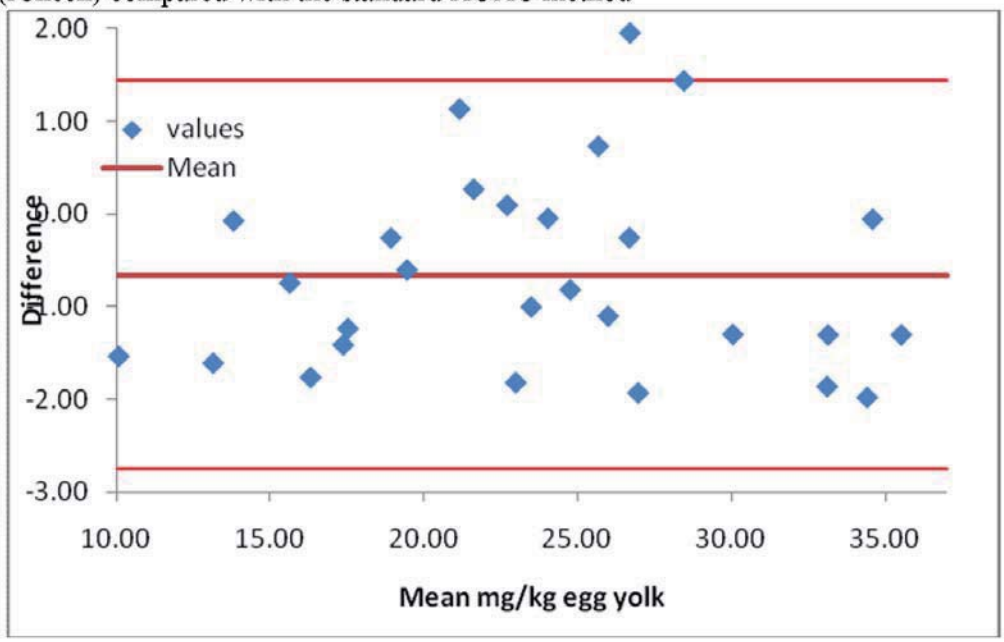

Table 1: Comparison of total carotenoids levels (mg/kg egg yolk) measured with the new method and with the standard AOAC method.

Group	iCheck	AOAC	% iCheck of AOAC
1	22.01±2.82	22.73 ± 3.30	97.2 ± 4.7
2	25.62 ± 6.78	26.45 ± 6.99	96.8 ± 3.0
3	22.88 ± 4.23	22.08 ± 3.68	103.5 ± 6.0
4	15.27 ± 5.47	16.40 ± 5.42	92.5 ± 5.2
5	30.84 ± 3.18	31.43 ± 4.17	98.5 ± 5.0
Sum	23.28 ± 6.73	23.77 ± 6.77	97.7 ± 5.8

Table 2: Comparison of total carotenoid contents (TCA) found by HPLC and iCheck in egg yolks containing apoester and lutein zeaxanthin in various concentrations

Carotenoid Contents Measured by HPLC (mg/kg)				iCheck Sample Portions (g)							
				0.8	0.4	0.2	0.1	0.8	0.4	0.2	0.1
LUT	ZEA	APO	TCA	TCA found by iCheck (mg/kg)				Deviation iCheck – HPLC for TCA (%)			
0.90	0.09	-	1.41	1.43	2.63			1.8	87		
0.94	0.11	2.99	4.33	4.66	5.95			7.5	38		
0.97	0.11	8.32	9.73	10.1	11.2			3.9	15		
1.01	0.11	16.7	18.2		19.6				7.7		
1.09	0.12	35.7	37.4		39.4				5.4		
1.09	0.13	71.1	72.9		77.1	75.8			5.8	3.9	
1.10	0.13	136	138			144				4.0	
1.13	0.14	271	273			300	303			9.6	11

See Table 3 for the legend

Table 3: Comparison of total carotenoid contents (TCA) found by HPLC and iCheck in egg yolks from the treatment groups P – X with canthaxanthin and various mixtures of apoester and canthaxanthin

Carotenoid Contents by HPLC (mg/kg)				Measured	iCheck Sample Portions (g)					
LUT	ZEA	APO	CAN	TCA	0.8	0.4	0.2	0.8	0.4	0.2
					TCA found by iCheck (mg/kg)			Deviation iCheck – HPLC for TCA (%)		
1.03	0.12		5.28	6.88	5.17	6.81		-25	-0.6	
1.06	0.12		20.1	22.4	15.9	17.8		-29	-21	
0.93	0.11	6.97	2.08	10.4	10.4	11.7		0.3	12	
1.17	0.12	8.96	5.68	16.4		16.4			0.4	
1.11	0.13	8.79	10.2	20.9		19.8			-5.3	
1.16	0.13	17.9	17.0	37.3		33.9			-9.1	
1.09	0.15	16.5	19.7	38.8		35.4			-8.5	
1.01	0.13	131	4.58	137			146			6.0
1.13	0.16	139	14.7	156			163			4.6

LUT = lutein, ZEA = zeaxanthin, APO = apoester, CAN = canthaxanthin, TCA = total carotenoid. In case of HPLC, TCA was determined as the sum of mentioned and non-identified carotenoids (contents not shown). For comparison with iCheck, the HPLC data was set to 100%. The shown contents are the means of 4 independent extractions and HPLC measurements.

Table 4: Comparison of total carotenoid contents (TCA) found by HPLC and iCheck in egg yolks from the treatment groups Y - AB with various concentrations of Tagetes xanthophylls

Carotenoid Contents Measured by HPLC (mg/kg)			iCheck Sample Portions (g)					
LUT	ZEA	TCA	0.8	0.4	0.2	0.8	0.4	0.2
			TCA found by iCheck (mg/kg)			Deviation iCheck – HPLC for TCA (%)		
6.18	0.46	8.86	6.88	9.30		-22	5.0	
6.80	0.54	9.73	8.24	9.37		-15	-3.7	
5.93	0.42	8.05	6.88	9.60		-19	19	
5.99	0.44	8.51	7.74	8.18		-9.1	-3.9	
6.23	0.47	8.79	7.43	9.11		-15	4.2	
21.9	1.87	30.9		27.9			-9.7	
22.6	1.69	30.6		29.0			-5.3	
24.6	1.73	32.6		29.4			-10	
22.9	1.82	32.3		31.8			-1.5	
23.0	1.78	31.6		29.5			-6.7	
50.8	4.05	69.7		58.6	60.2		-16	-14
50.0	3.77	69.2		60.4	64.0		-13	-8
49.0	3.96	68.0		58.7	62.7		-14	-8
43.0	3.58	60.1		52.2	50.8		-13	-16
48.2	3.84	66.8		57.5	59.4		-13	-11
75.6	6.01	109		111	109		1.5	0.2
72.0	5.50	104		92.7	93.8		-11	-9.5
72.3	5.71	102		90.9	96.8		-11	-5.1
75.1	5.73	106		92.3	99.2		-13	-6.7
73.8	5.74	105		96.6	99.8		-5.3	-8.3

See Table 3 for the legend