TECHNICAL COMMENT

Effects of Temperature and Duration of Storage on the Stability of Antioxidant Compounds in Egg Yolk and Plasma

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ABSTRACT

Antioxidants help protect tissues from oxidative damage caused by reactive oxygen species. In view of the widespread interest in egg yolk and plasma antioxidants in relation to environmental and life-history variables, there is a need for knowledge on the necessary conditions for sample storage, which is currently lacking. In this study, our purpose was to examine the kinetics of the degradation of lutein, retinol, and tocopherol in egg yolk samples and the total antioxidant capacity in plasma samples stored at three different temperatures (−20°, −50°, and −80°C) for 24 mo. We found that yolk lutein was stable during the study period. Yolk retinol and tocopherol showed a steep early decline and then remained relatively stable, but retinol showed significant losses at the end of the study period too. In contrast to our expectations, there was no difference in the stability of antioxidant compounds of egg yolk samples stored at different temperatures. Plasma OXY level was stable during the first 6 mo, showed a slight decline between 6 and 12 mo, and declined more intensely after 12 mo of storage. We suggest that studies focusing on the analysis of egg yolk retinol or tocopherol concentrations and total plasma antioxidant capacity should analyze the samples in the first 6–7 mo after collection. For the analysis of yolk lutein, samples can be stored for 24 mo without significant degradation. The storage temperature of −20°C seemed to be sufficient, as a lower temperature did not significantly affect the slope of degradation of the samples.

Keywords: carotenoids, degradation, lutein, OXY test, total antioxidant capacity, vitamins.

Introduction

During aerobic metabolism, large amounts of free radicals and reactive oxygen species (ROS) are produced (Halliwell and Gutteridge 2007). Intense ROS production can be generated by rapid growth, reproduction, intense metabolism, infection, or metal accumulation (Costantini 2008; Koivula and Eeva 2010; Metcalfe and Alonso-Alverez 2010). ROS are highly unstable and can cause oxidative damage to DNA, proteins, lipids, and other molecules, which can result in loss of cell homeostasis and function (Halliwell and Gutteridge 2007). Accumulation of oxidative damage is supposed to contribute to cellular senescence and play an important role in aging and the cost of reproduction, as well as many diseases and disorders (Finkel and Holbrook 2000; Costantini 2008; Metcalfe and Alonso-Alverez 2010). A high level of ROS production or a decreased efficiency of the antioxidant system can lead to oxidative stress (Halliwell and Gutteridge 2007; Costantini and Verhulst 2009).

The antioxidant defense system of an organism includes endogenous, synthesized molecules (e.g., enzymes or repair system) and exogenous, ingested antioxidants (e.g., vitamins A and E, carotenoids). Antioxidants can also be classified as hydrophilic (e.g., ascorbic acid, polyphenols) and lipophilic (e.g., carotenoids, tocopherol; Surai 2002; Halliwell and Gutteridge 2007). A large number of oviparous species allocate micronutrients with antioxidant properties to the egg yolk, which is thought to be an important maternal effect to help protect the embryonic tissues from oxidative damage and thus improve the viability of the young (Mousseau and Fox 1998; Blount et al. 2000). Carotenoids, tocopherols, and retinol are important lipid-soluble antioxidants the female birds acquire from the diet and allocate to the egg yolk (Olson 1993; Woodall et al. 1996; Surai et al. 1999; Surai 2002). These compounds help protect the vulnerable, lipid-rich tissues of bird embryos from the attack of free radicals and ROS, which are by-products of rapid oxidative metabolism (Halliwell and Gutteridge 2007).

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In behavioral ecological studies, there is a high interest in the relationship between various environmental and life-history variables and the antioxidant concentration of the egg yolk (Saino et al. 2002; McGraw et al. 2005; Navara et al. 2006; Török et al. 2007; Isaksson et al. 2008; Remeš et al. 2011) and the antioxidant capacity of the blood (Costantini et al. 2006, 2007; Costantini 2010; Markó et al. 2011; Saino et al. 2011). Studies rely on assays of egg yolk and plasma samples that have generally been stored for long periods in a freezer. In view of the widespread study of egg yolk and plasma antioxidants, there is a need for knowledge of the necessary conditions for sample storage, which is currently lacking. It is possible that during long-term freezer storage, the levels of antioxidants decrease. This possibility is an important concern, as micronutrients may degrade below the detection limit of an assay or concentration values might become unreliable.

It is generally reported that yolk and plasma samples were stored at −20°C, but the duration of storage is rarely presented. Researchers often do not have the facility, the laboratory skills, or the financial resources to complete the analysis of antioxidant concentrations of their samples immediately after collection. Therefore, samples are often stored long-term in a freezer until later analysis. It would be important to know whether degradation of antioxidants in egg yolk and plasma can be slowed down if samples are stored at lower temperature. Moreover, knowledge on the duration until which the samples can be stored without considerable reduction in their antioxidant concentrations is also crucial, especially in studies of wild animals.

In this study, our purpose was to examine the kinetics of the degradation of lipophilic antioxidants in egg yolk, such as carotenoid (lutein), tocopherol, and retinol, and the total antioxidant capacity of plasma samples stored at three different temperatures (−20°, −50°, and −80°C). We predicted that degradation would be slower when samples are stored at lower temperature. To our knowledge, no study has examined the effect of storage conditions on the antioxidant concentrations of unprocessed egg yolk. Moreover, the total plasma antioxidant capacity is also an important variable indicating the quality of the individual, which has been used in a great number of recent studies (Costantini et al. 2006, 2007; Costantini 2010; Markó et al. 2011; Saino et al. 2011). Again, it has not been documented whether samples can be stored long-term and what the ideal freezing temperature would be to avoid reduction in total antioxidant capacity.

Methods

Egg Yolk Preparation

Ten hen eggs of the same size and quality were acquired from a local supermarket on the same day and stored for 5 d at 4°C. Then, the eggs were opened, the white, yolk, and shell separated, and egg yolks homogenized. Each egg yolk was divided into 3 × 5 aliquots of 120–200 mg yolk. Each group of five aliquots was put into a freezer of different temperature (−20°, −50°, and −80°C). Egg yolks stored in the three types of freezers were analyzed by high-pressure liquid chromatography (HPLC) within 10 d (0 mo) and after 7, 12, 19, and 24 mo, each time using another aliquot. The results of the first measurement (0 mo) were averaged for each egg yolk, and those values were used as the first data points in each series. Repeatabilities of concentration values of the first measurement were 77.5% for lutein ($F_{9,16} = 9.90$, $P < 0.001$), 59.4% for retinol ($F_{9,13} = 4.33$, $P = 0.009$), and 45.8% for tocopherol ($F_{9,12} = 2.83$, $P = 0.048$).

Plasma Preparation

Eleven blood samples (30–60 μL) were taken into heparinized capillary tubes from the brachial veins of female great tits (*Parus major*), breeding in a nestbox plot in Pilis Mountains, Hungary, in 2012. Capillary tubes were centrifuged at 10,000 rpm for 10 min on the same day, and plasma and red blood cells were separated. Plasma samples were stored at −50°C for 1 mo, when the first measurement by the OXY-Adsorbent test (Diacron, Grosseto, Italy) was conducted. Then, plasma samples were divided into three aliquots of 5–15 μL plasma. Each aliquot of the same plasma was stored in Eppendorf tubes closed by a paraffin film in a freezer of different temperature (−20°, −50°, and −80°C). Plasma samples stored at the three types of freezers were analyzed by OXY-Adsorbent test after 6, 12, 18, and 24 mo of blood collection, each time repeatedly using the same aliquot in the freezer. Samples stored at −20° and −50°C containing less than 8 μL plasma ($n = 4$ in both temperatures) were analyzed only after 12 and 24 mo.

Egg Yolk Antioxidant Analysis by HPLC

Egg yolk antioxidant concentration was analyzed by HPLC as previously described in Hargitai et al. (2010), with some slight modifications. First, an internal standard (astaxanthin) was added to the yolk sample. The sample was placed in a mortar containing 500 mg C_{18} sorbent, and it was manually blended. The blended material was then transferred into a column suitable for conducting sequential elution and pressed to a compact column bed between two frits. The column was then washed with 3 mL water to elute the polar impurities. Before eluting the analytes, a second column packed with sodium sulfate was placed under the first column in order to remove the water residue from the effluent. The carotenoid fraction was eluted with 8 mL of dichloromethane. After evaporating the solvent to dryness under a nitrogen stream at 40°C, the residue was redissolved in 200 μL dichloromethane : methanol (1 : 4) solution. A reversed-phase Develosil C_{30} (150 mm × i.d. 4.6 mm, 3 μm) column was used with a Zorbax C_{18} (12.5 mm × i.d. 4.6 mm, 5 μm) precolumn in an HP 1050 system. A 10-μL sample was injected onto the column and eluted with a gradient starting at 10% water (eluent A) and 90% acetonitrile : ethyl acetate (2 : 1) with 0.05 % (v/v) triethylamine (eluent B). This composition was kept for 3 min with a flow rate of 0.5 mL/min, after which it was changed to 100% B over a period of 6 min. This final composition was kept for 11 min with a flow rate of 1.0 mL/min for 4 min and 1.5 mL/min for 7 min. The column was kept at

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50°C. The UV-VIS detector was operated at 450 nm (lutein), 325 nm (retinol), and 295 nm (tocopherol). Peaks were identified and quantified by comparison with retention times and peak areas of lutein (CaroteNature), retinol, and tocopherol (Sigma) standards.

Plasma OXY Test

Total plasma antioxidant capacity was measured by the OXY-Adsorbent test. This test measures the ability of the antioxidant compounds of the plasma (e.g., carotenoids; vitamins A, C, and E; proteins; thiols) to cope with the oxidant action of hypochlorous acid (HClO). First, 2 μL of plasma was diluted 1 : 100 with distilled water. Then, 200 μL of HClO solution was pipetted into the wells of a microplate and 2 μL of the diluted samples was added to each well. The microplate was then incubated at 37°C for 10 min with shaking at medium intensity. After that, 2 μL of a chromogenic mixture was added to the wells. The alkyl-substituted aromatic amin solubilized in the chromogen was oxidized by the residual HClO of the solution and thus transformed into a pink derivative. Accordingly, the intensity of the pink color is inversely related to the total antioxidant capacity of the plasma. After 5 min of shaking at medium intensity, the absorbance was read by a spectrophotometer (BioTek ELX808 with Gen5 software) at 540 nm. Absorbances were compared to those of a dilution series of a calibrator (stabilized protein solution). Samples were tested in duplicates (mean CV% = 1.6%), and results were expressed as millimolars HClO neutralized. Moreover, we measured five plasma samples stored at −80°C five times (days 1, 6, 10, 14, 19) during a period of 19 d to test whether a repeated freezing/thawing could cause a significant deterioration in the level of total plasma antioxidant capacity.

Statistical Analysis

We conducted general linear mixed models with sample (egg yolk or plasma) identity as a random factor. We entered duration of storage as a continuous predictor variable and storage temperature as a three-level factor. Interactions between predictor variables were also included. We calculated standardized effect sizes as $R = \{(F \times \mathrm{df1})/[(F \times \mathrm{df1}) + \mathrm{df2}]\}^{1/2}$ (McNeil et al. 1996). When the effect of storage duration was significant, we repeated the analysis including storage duration as a five-level factor in order to conduct pairwise comparisons with Bonferroni adjustment for multiple comparisons. In all models, a stepwise analysis based on backward deletion procedure was employed, removing nonsignificant ($P > 0.05$) effects from the model in decreasing order of P value. Analyses were performed in SPSS 19.0 (SPSS, Chicago, IL), and figures were prepared in STATISTICA 5.5 (StatSoft, Tulsa, OK).

Results

We found no effect of storage temperature (−20°, −50°, and −80°C) on the stability of egg yolk lutein, retinol, and tocopherol (lutein: duration × temperature interaction, $F_{2, 138.0} = 1.76$, $P = 0.18$; temperature, $F_{2, 138.0} = 0.67$, $P = 0.52$; retinol: duration × temperature interaction, $F_{2, 136.0} = 1.11$, $P = 0.33$; temperature, $F_{2, 136.1} = 0.19$, $P = 0.83$; tocopherol: duration × temperature interaction, $F_{2, 133.9} = 0.39$, $P = 0.68$; temperature,

Table 1: Change in the mean concentration (±SD) of lutein, retinol, and tocopherol in hen egg yolks after storage for specified durations at −20°, −50°, and −80°C, respectively

	−20°C			−50°C			−80°C		
Component and storage duration (mo)	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
Yolk lutein (μg/g):									
0	15.75	5.42	10	15.75	5.42	10	15.75	5.42	10
7	13.29	4.20	10	15.77	5.62	9	10.87	3.27	10
12	17.10	5.33	10	16.54	4.93	10	16.76	4.96	10
19	16.80	5.02	10	15.48	5.24	10	18.33	3.10	9
24	13.72	4.42	10	14.43	3.96	10	14.62	4.94	10
Yolk retinol (μg/g):									
0	12.97	2.69	10	12.97	2.69	10	12.97	2.69	10
7	8.01	1.63	10	8.00	1.62	8	6.82	1.23	10
12	8.99	1.82	10	8.95	1.39	10	9.14	1.58	10
19	6.70	1.66	10	6.35	1.87	10	8.69	1.19	8
24	4.11	1.06	10	5.20	1.10	10	4.67	1.26	10
Yolk tocopherol (μg/g):									
0	88.69	21.07	10	88.69	21.07	10	88.69	21.07	10
7	59.00	20.28	10	65.51	28.72	8	61.80	15.99	10
12	63.55	20.68	9	70.17	27.11	10	74.22	28.82	10
19	59.28	16.39	10	53.17	15.66	10	59.98	25.01	9
24	62.50	15.54	9	71.56	17.58	9	51.96	18.55	10

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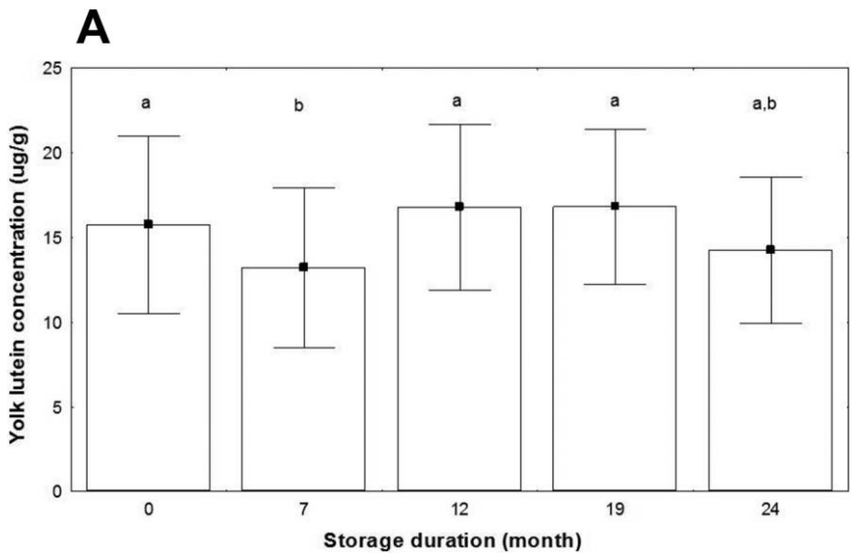

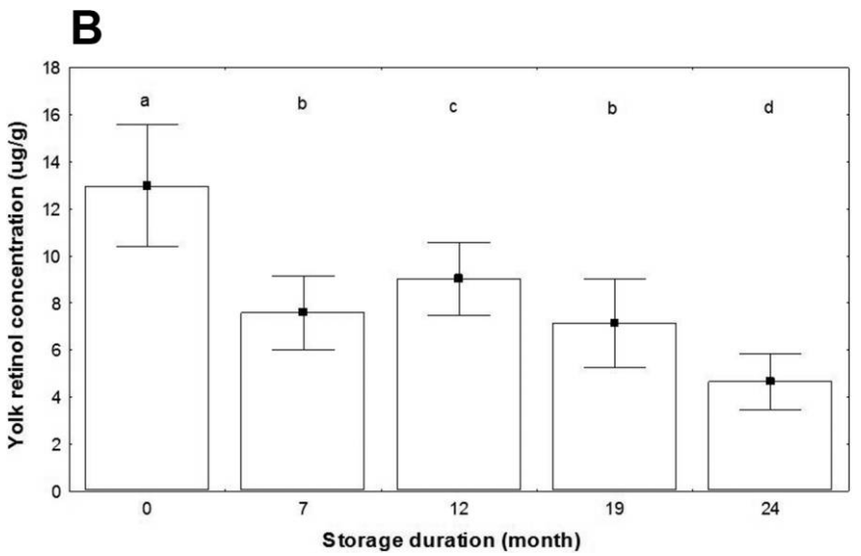

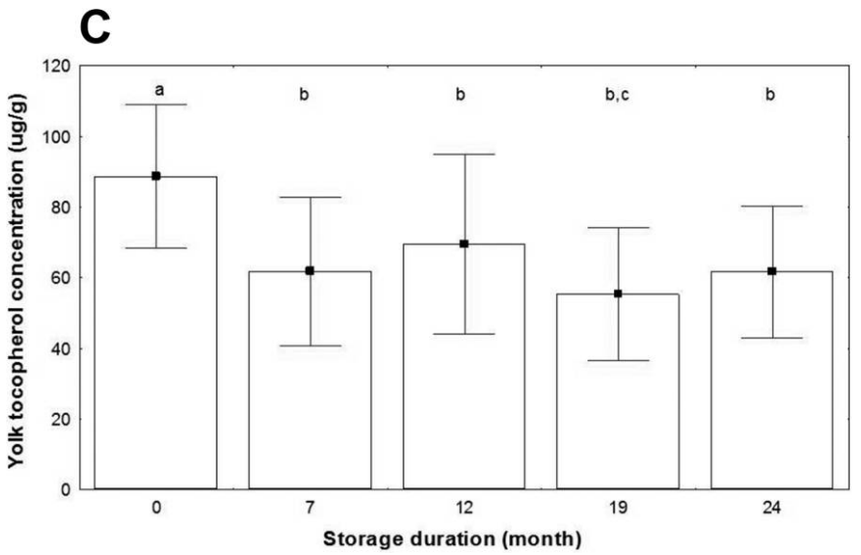

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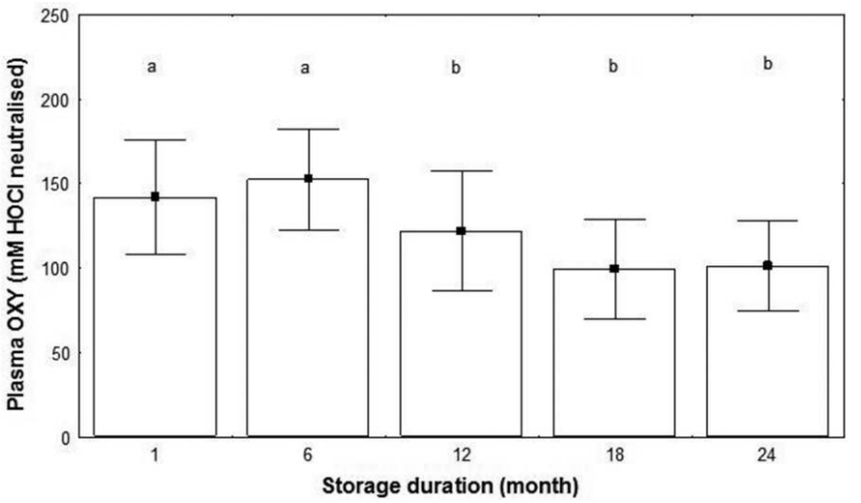

Figure 2. Effect of storage duration (month) on mean concentration of total antioxidant capacity (OXY) of great tit plasma. Values are means (±SD) of samples stored at −20°, −50°, and −80°C. Values not sharing the same letter are different at $P < 0.05$ in pairwise comparisons with Bonferroni adjustment for multiple comparisons.

$F_{2, 133.9} = 1.54$, $P = 0.22$). The three lipid-soluble antioxidant components of the yolk showed differences in their stability in relation to storage duration (table 1). Lutein was the most stable component, which remained almost constant during the study period (duration, $F_{1, 138.0} = 0.00$, $P = 0.98$; fig. 1*A*) and declined on average to 91% after 24 mo of storage (table 1).

However, egg yolk retinol and tocopherol concentrations showed a significant decline during the study period (retinol, $F_{1, 136.0} = 283.1$, $P < 0.001$, $R = -0.82$; fig. 1*B*; tocopherol, $F_{1, 134.0} = 66.10$, $P < 0.001$, $R = -0.57$; fig. 1*C*). Egg yolk retinol and tocopherol concentrations showed a steep early decline and then remained relatively stable, but retinol showed significant losses at the end of the study period too (table 1). Retinol concentration declined on average to 70% after 12 mo and to 36% after 24 mo of storage, while tocopherol concentration declined on average to 78% after 12 mo and to 67% after 24 mo of storage (table 1).

Plasma OXY level was not affected by storage temperature (duration × temperature interaction, $F_{2, 138.0} = 0.32$, $P = 0.73$; temperature, $F_{2, 138.1} = 1.88$, $P = 0.16$). It also showed a slow degradation ($F_{1, 138.0} = 103.23$, $P < 0.001$, $R = -0.65$; fig. 2): until 6 mo of storage, no significant reduction was detected; then a slight decline on average to 86% was shown after 12 mo of storage (table 2). After 12 mo, a more considerable reduction was observed: plasma OXY level declined on average to 71% after 24 mo of storage (table 2). We found that plasma OXY level showed an increase with the repeated cycles of thawing and freezing ($F_{1, 19.1} = 19.44$, $P < 0.001$, $R = 0.71$). The detailed pairwise comparisons showed that plasma OXY level was not statistically different between two consecutive measurements (all $P > 0.61$); only the last (fifth) measurement value was statistically higher than the first two values (1–5, $P = 0.004$; 2–5, $P = 0.016$; all other $P > 0.23$).

Discussion

In our study, we found that egg yolk lutein concentration was stable during the 24-mo study period. Egg yolk retinol and tocopherol concentrations showed a steep decline during the early storage period (a loss of 42% and 29%, respectively), and then they remained relatively stable, although retinol showed significant losses at the end of the study period too. In contrast to our expectations, there was no difference in the stability of lutein, retinol, and tocopherol in yolk samples stored at −20°, −50°, or −80°C.

Studies investigating the storage stability of antioxidants in egg yolk are almost lacking. Similarly to our results, Hauge et al. (1944) found that storage for 12 mo at −18°C caused no significant loss in carotenoid levels of homogenized liquid and dried eggs. In contrast, in a study of freeze-dried egg yolk powder, it was found that lutein and zeaxanthin levels showed a loss of 59%–69% after 6 mo of storage at −18°C (Wenzel et al. 2011). In studies of plasma or serum samples, it was reported that carotenoid, tocopherol, and retinol were stable for at least 28 mo at −70°C (Matthews-Roth et al. 1984; Craft et al. 1988; Comstock et al. 1995). At −20°C, retinol and tocopherol in plasma samples were stable for at least 15 mo (Driskell et al. 1985; Craft et al. 1988; Gunter et al. 1988). However, lutein

Figure 1. Effect of storage duration (month) on mean concentration of lutein (*A*), retinol (*B*), and tocopherol (*C*) of hen egg yolks. Values are means (±SD) of samples stored at −20°, −50°, and −80°C. Values not sharing the same letter are different at $P < 0.05$ in pairwise comparisons with Bonferroni adjustment for multiple comparisons.

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Table 2: Change in the mean concentration (±SD) of total antioxidant capacity (OXY) in great tit (*Parus major*) plasma after storage for specified durations at −20°, −50°, and −80°C

	−20°C			−50°C			−80°C		
Component and storage duration (mo)	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
Plasma OXY (mM HClO neutralized):									
1	141.92	35.04	11	141.92	35.04	11	141.92	35.04	11
6	146.48	30.03	7	152.63	20.57	7	156.58	35.98	11
12	118.59	31.86	11	118.46	45.85	11	128.44	29.29	11
18	105.77	25.01	7	71.78	31.97	7	113.17	18.39	11
24	95.49	33.20	11	102.57	27.61	11	105.72	19.32	11

concentration in plasma samples stored at −20°C decreased by 15% after 6 mo (Matthews-Roth et al. 1984) and by 24% after 15 mo of storage (Craft et al. 1988). These results suggest that tocopherol and retinol are considerably stable in frozen blood samples, but carotenoids are less stable, and storage temperature affects the stability of carotenoids.

It is difficult to find an explanation for the inconsistency between earlier results on plasma samples and our results on egg yolk. Egg yolk is a lipophilic matrix containing large amounts of lipoproteins and a relatively high concentration of antioxidants (Huopalahti et al. 2007). It is possible that in unprocessed frozen egg yolk, the integration of the yolk matrix and binding to lipoproteins may stabilize carotenoids for a longer time. Moreover, tocopherol may have a protective effect on carotenoids (Palozza and Krinsky 1992; Böhm et al. 1998; Stahl et al. 2000), possibly reducing the oxidative degradation of carotenoids in the yolk.

Our results showed that plasma OXY level was not affected by storage temperature, but it showed a slow decline with storage duration. Until 6 mo of storage, no significant reduction was detected; between 6 and 12 mo of storage, it showed a slight decline; and after that time, a stronger reduction was detected, leading to 71% after 24 mo of storage. Plasma samples were repeatedly frozen and thawed for the analysis of OXY, which might also cause some decline in the plasma antioxidant capacity. However, a number of prior studies have shown no demonstrable effect on concentration of several antioxidants in plasma of repeated freeze-thaw cycles (Comstock et al. 1995). Furthermore, we found that repeated freezing/thawing caused a significant increase in plasma antioxidant capacity, which could be explained by plasma samples in Eppendorf tubes becoming more concentrated after several instances of thawing, opening, and refreezing. Therefore, our results suggest that repeated freeze-thaw cycles do not cause a significant deterioration in the level of total plasma antioxidant capacity. As we have used the same Eppendorf tubes for the analyses of OXY, it is possible that OXY level declined more intensely by the end of the study period but the increase due to the repeated freezing/thawing masked this intense decline.

Overall, our data suggest that studies focusing on the analysis of egg yolk retinol and tocopherol should analyze the yolk samples in the first 7 mo after collection to avoid a significant reduction in their concentrations. Carotenoid (lutein) concentration seemed to be stable and did not decline significantly during the 24-mo study period. This result suggests that for a study investigating yolk carotenoid concentration, samples can be stored without a significant decrease for at least 24 mo. For the analysis of total antioxidant capacity of plasma samples, measurement in the first 6 mo after collection is recommended. The storage temperature of −20°C seemed to be sufficient, as storing the samples at −50° or −80°C did not significantly slow the reduction of the antioxidant concentrations.

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