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METHODS

A quantitative comparison of the commonly used methods for extracting carotenoids from avian plasma

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Abstract Interest in animal carotenoids, especially in birds, has exploded in recent years, and so too have the methods employed to investigate the nature and function of these pigments. Perhaps the most easily and commonly performed procedure in this work has been the determination of carotenoid concentration from avian plasma. Over the past 20 years of research on avian carotenoids, numerous methods have been used to extract carotenoids from bird plasma, all of which have differed in several important parameters (e.g., number and types of solvents used, degree of mixing/centrifugation). However, to date, no study has systematically compared these methods to determine if any of them are more effective than others for recovering any or all types of carotenoids present. We undertook such an investigation on plasma samples from two bird species (house finch, *Carpodacus mexicanus*, and mallard, *Anas platyrhynchos*) using five of the most commonly employed methods for extracting carotenoids from avian plasma: (1) acetone-only, (2) methanol-only, (3) ethanol-only, (4) ethanol + hexane, and (5) ethanol + *tert* butyl methyl ether. We also manipulated the amount of time that extracts were centrifuged, which has varied tremendously in previous studies, to evaluate its importance on carotenoid recovery. We found that all methods equally recovered the polar xanthophylls (lutein and zeaxanthin), but that the methanol-only procedure poorly recovered non-polar carotenoids (less β-carotene in both species and less β-cryptoxanthin in house finches) compared to the other methods. These results suggest that the data accumulated to date on xanthophyll plasma carotenoids in birds should be comparable across studies and species despite the different chemical extraction methods used. However, care should be taken to use relatively strong organic solvents for fully recovering non-polar carotenoids. We also found no effect of centrifugation duration (1 vs. 10 min at 10,000 rpm) on carotenoid recoveries, demonstrating that researchers can save considerable time by centrifuging for a much shorter time period than is typically used.

Keywords Carotenoid pigments · Ethanol · House finch · HPLC · Lutein · Mallard · Methanol · Zeaxanthin

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Introduction

Carotenoid pigments have recently captured the attention of biologists because of their unique and wide-ranging controls and functions in animals (Vershinin 1999). From sea urchins to birds, carotenoids play key roles in health, mating, breeding, and development (McGraw 2006a). This is typically because carotenoids are a dietarily (Grether et al. 1999; Hill et al. 2002), physiologically (McGraw and Parker 2006; Blas et al. 2006), or immunologically (Blount et al. 2003a; McGraw and Ardia 2003) limiting but valuable resource in animals. A major challenge to ecologists and evolutionary biologists interested in carotenoids has been to biochemically track these lipid-soluble pigments in foods, flesh, feathers, and other tissues (Hudon and Brush 1992; Stradi et al. 1995) so that we may determine their limitations and allocation priorities.

One of the easiest and most common ways to determine the carotenoid status of vertebrates has been to assay the carotenoid content of blood plasma or serum, which represents the current pool of pigments (ingested from food

and/or retrieved from tissue stores) available for allocation to various body functions (e.g., oxidative stress, immune challenges, color acquisition/maintenance, yolk formation). Particularly in birds, numerous researchers have used solvent extraction techniques to isolate carotenoids from serum/plasma of wild animals with the aim of comparing such levels to factors such as diet, health, age, phylogeny, coloration, and tissue carotenoid concentrations (Blount et al. 2002; Tella et al. 2004; McGraw et al. 2006a; Costantini et al. 2007a; Martinez-Padilla et al. 2007). However, this has been done using a variety of chemical extraction methods, none of which have been quantitatively compared to date to determine their relative effectiveness. A survey of recently published papers reveals that five different procedures have mainly been used to extract carotenoids from bird plasma (Appendix), which differ primarily in the type (s) of solvent(s) used. Most common among these is an acetone-only extraction first used by Ruff et al. (1974) and Allen (1987a, b) with chickens. The next two most frequently used methods involve a two-step procedure where ethanol is used first to remove protein and then a second organic solvent—either hexane (adapted from extraction methods on human plasma carotenoids, i.e., Staciewicz-Sapuntakis et al. 1987) or *tert* butyl methyl ether (TBME; originally employed by McGraw et al. 2002 with zebra finches)—is added to fully recover lipid-soluble carotenoids. The fourth and fifth methods have been recently added as single-solvent extractions using either ethanol or methanol. All of these solvents differ considerably in their polarity and thus their ability to solubilize different types of carotenoids (e.g. polar xanthophylls versus non-polar cryptoxanthins and carotenes). If these methods were to differ significantly in their carotenoid recoveries, attempts to draw comparisons among types and amounts of plasma carotenoids across studies and species would be difficult. Thus, a systematic, quantitative comparative study of plasma carotenoid extraction methods in birds is warranted.

We undertook such an investigation by comparing carotenoid recoveries among five aforementioned carotenoid extraction methods using plasma from two free-ranging bird species from North America—the house finch (*Carpodacus mexicanus*) and the mallard (*Anas platyrhynchos*). We performed this test on these two species as an attempt to understand the generality of the results and because plasma in both species were known to contain both polar and non-polar carotenoids (McGraw et al. 2006a; M. W. Butler and K. J. McGraw, unpublished data). We could have tested numerous procedural variables in this study (e.g., degree of mixing between solvent and plasma, duration of plasma/extract storage prior to analysis), but instead focused on the two factors that seemed to show the highest and (to us) most important variability in the literature: (1) solvent type and (2) centrifugation time. We hypothesized that methods using particularly weak solvent types, like ethanol and methanol, would poorly recover carotenoids, especially non-polar forms, from plasma compared to the other three methods. Consistent with this, a previous study that compared solvent extraction methods for plant carotenoids showed that methanol failed to fully recover β-carotene (Dunn et al. 2004). We also hypothesized that longer centrifugation times could result in the loss of carotenoids (if carotenoids are not wholly soluble in some solvents and thus are spun down with the pellet) or add unnecessary processing time for researchers if, for example, 1 min as opposed to ten is sufficient for centrifuging the solutions (to remove flocculant proteins). It is noteworthy that a few other methods for extracting avian plasma have been employed, but only once in the literature (acetonitrile-only: Saino et al. 1999 in barn swallows; hexane-only: Zhao et al. 2006 in chickens; ethanol + ethyl ether: Khachik et al. 2002 in quail; three-step organic solvent extraction process: Koutsos et al. 2003a, b in chickens; ethanol/chloroform/methanol: Wang et al. 2007 in chickens), and thus for the sake of feasibility, we did not include them here.

Materials and methods

We used heparanized capillary tubes to collect 80–200 μl blood from 50 wild-caught house finches in Tempe, AZ on 20 May 2007 and to collect 120–320 μl blood from 30 wild-caught mallard ducklings in Tempe, AZ from May to June 2007. We centrifuged the blood at 10,000 rpm for 2 min and saved plasma fractions in 1.5-ml screw-capped Eppendorf tubes at −80°C for 4–6 weeks until analysis. To obtain large enough samples on which we could conduct controlled experimental tests, we pooled plasma samples from four to six individuals to generate nine stock samples of mallard plasma and 11 stock samples of house finch plasma, each containing approximately 200 μl plasma. These stocks were vortexed thoroughly at the original time of mixing and before each test aliquot was drawn from it to ensure homogenization.

A 5×2 factorial design was used to extract carotenoids from each of the stock plasma samples: five solvent combinations (see above and Appendix) and two centrifugation durations (1 and 10 min, both at 10,000 rpm). We chose 10,000 rpm as a standard speed because of recent trends in the literature by other authors (publications from 2004–present in Appendix), because of previous work by the first author (McGraw 2005), and because it should work more effectively than slower speeds (e.g., Bortolotti et al. 1996). We chose these two durations for centrifugation because 10 min is the typical time allotted for this step in the literature (Appendix) and because we recently began trying

Table 1 Mixed-model ANOVA table depicting the effects of solvent extraction method, centrifugation time, and their interaction on the recovery of different carotenoids from the plasma of wild house finches

Significant terms are in bold.

Source	Lutein	Zeaxanthin	β-cryptoxanthin	β-carotene
Solvent extraction method	$F_{4,90}$=1.98 P=0.10	$F_{4,90}$=1.89 P=0.12	$F_{4,63}$=**4.54** P=**0.003**	$F_{4,81}$=**14.05** P<**0.0001**
Centrifugation time	$F_{1,90}$=1.29 P=0.26	$F_{1,90}$=0.89 P=0.35	$F_{1,63}$=1.50 P=0.23	$F_{1,81}$=0.56 P=0.46
Solvent method × centrifugation time	$F_{4,90}$=0.35 P=0.84	$F_{4,90}$=0.65 P=0.63	$F_{4,63}$=0.82 P=0.51	$F_{4,81}$=0.14 P=0.97

shorter spin durations such as 1 min with no apparent ill effects on carotenoid recovery (personal observations). Samples from each stock were all extracted and run (using high-performance liquid chromatography; see more below) simultaneously to avoid inter-assay variation; within each assay, we also randomized the order in which samples from different treatments were processed to ensure no sequence biases. Into each of ten different tubes for each stock, we transferred 15 μl plasma, followed by 150 μl of the appropriate solvent(s). A 1:10 dilution (plasma-to-each solvent) was used throughout because this is the most frequently reported ratio in the literature (Appendix). We vortexed tubes for 5 s after each solvent was added, centrifuged them for their allotted times (in a Beckman-Coulter Microfuge® 18 centrifuge, Fullerton, CA, USA), and then transferred each solution to a fresh tube. Solvents were immediately evaporated to dryness under a stream of nitrogen and samples prepared for analysis via high-performance liquid chromatography (HPLC).

Following prior work by the first author (McGraw et al. 2006a), we used a Waters Alliance HPLC instrument equipped with a Waters Carotenoid C-30 column to determine types and amounts of carotenoids present. The gradient method previously employed (McGraw et al. 2006a) was modified slightly to cut down on run times: initial isocratic conditions were maintained until minute 11, at which point we began running the linear gradient until minute 21. Final gradient conditions were then held until minute 25 and then returned to initial isocratic conditions until minute 29.5. Pigment concentrations were calculated based on external curves constructed from known amounts of purified reference carotenoids.

We extracted and ran 35% of all samples in duplicate to calculate coefficients of variation for the two carotenoids that consistently appeared in all samples, lutein and zeaxanthin. The overall coefficient of variation among all duplicate runs was 6% for lutein and 11% for zeaxanthin, the difference in them being mostly due to lower mean values (denominators) for zeaxanthin. Degrees of assay variation did not differ as a function of solvent extraction method or centrifuge time, nor did absolute carotenoid recoverability [which we tested using an internal standard, anhydrolutein, and which averaged 88.5±2.2%; two-way analyses of variance (ANOVAs), all P>0.13].

We compared carotenoid recoveries using mixed-model ANOVA, with solvent method, centrifugation time, and their interaction as fixed factors and sample as a random factor. Analyses were run using SAS 9.1 software for Windows (SAS Institute, Cary, NC, USA). Separate models were run for each carotenoid type in each species. We made post hoc pairwise comparisons among methods using Tukey's honestly significant difference (HSD) tests.

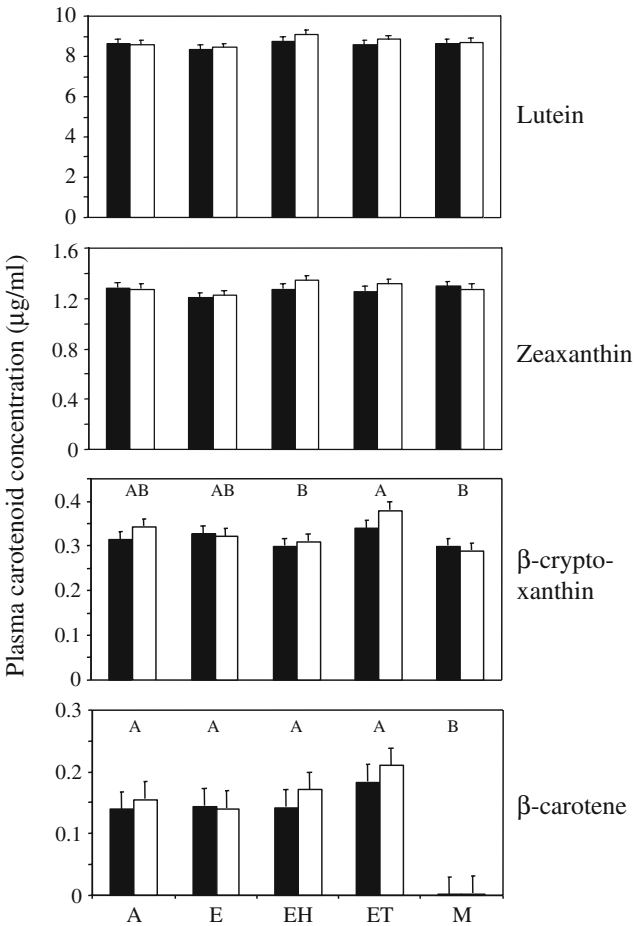

Fig. 1 Bar charts depicting carotenoid recoveries (means+SE) for different solvent extraction methods and centrifuge times in house finch plasma. *A* acetone, *E* ethanol, *EH* ethanol + hexane, *ET* ethanol + TBME, *M* methanol. *Dark bars* denote extracts that were centrifuged (to remove flocculant protein) for 1 min; *light bars* signify samples spun for 10 min. *Unshared letters atop the panels* (e.g., β-carotene) denote significant differences (p<0.05) among solvent types using Tukey's HSD tests

Table 2 Mixed-model ANOVA table depicting the effects of solvent extraction method, centrifugation time, and their interaction on the recovery of different carotenoids from the plasma of wild mallard ducklings

Significant terms are in bold

Source	Lutein	Zeaxanthin	β-cryptoxanthin	β-carotene
Solvent extraction method	$F_{4,72}$=1.7 P=0.16	$F_{4,72}$=0.96 P=0.44	$F_{4,45}$=0.54 P=0.71	$F_{4,18}$=**13.74** P<**0.0001**
Centrifugation time	$F_{1,72}$=0.58 P=0.45	$F_{1,72}$=1.12 P=0.29	$F_{1,45}$=0.11 P=0.74	$F_{1,18}$=0.45 P=0.51
Solvent method × centrifugation time	$F_{4,72}$=0.72 P=0.58	$F_{4,72}$=0.38 P=0.82	$F_{4,45}$=0.85 P=0.50	$F_{4,18}$=0.22 P=0.92

Results

House finch and mallard plasma during spring contained four main carotenoid types: lutein, zeaxanthin, β-cryptoxanthin, and β-carotene (McGraw et al. 2006a). Lutein was the dominant plasma pigment in house finches (approximately 80% of total), followed by zeaxanthin (approximately 10% of total), and both were present in every sample. These were accompanied by small amounts of β-cryptoxanthin (present in all but three samples) and β-carotene (present in all but one sample). Lutein (approximately 66% of total) and zeaxanthin (approximately 25% of total) were also dominant in mallard duckling plasma, and while β-cryptoxanthin and -carotene combined to make up a similar remaining amount of total plasma carotenoids compared to house finches, they were more often absent from mallard plasma (from three and six of the samples, respectively). The mean and range of total carotenoid concentrations were slightly higher in mallard samples (11.3 μg/ml and 4.4–34.1 μg/ml, respectively) than in house finch samples (10.3 μg/ml and 2.2–28.8 μg/ml, respectively).

The five solvent extraction methods employed did not differ significantly in the recovery of the two polar xanthophylls, lutein and zeaxanthin, in either finches (Table 1, Fig. 1) or mallards (Table 2, Fig. 2). However, we did find significant differences among methods in the recovery of β-cryptoxanthin in house finches and of β-carotene in both species (Tables 1 and 2). Methanol recovered significantly less of these carotenoids than all other methods (Figs. 1 and 2). In fact, methanol failed to recover any β-carotene in any mallard sample and only recovered trace amounts (0.01 μg/ml) in two of the 11 house finch samples. In addition, in house finch plasma, methanol and ethanol–hexane recovered significantly less β-cryptoxanthin, and ethanol–TBME significantly more, than the other methods. We failed to find any effect of centrifugation duration on carotenoid recoveries for any pigment type in either species (Tables 1 and 2).

Discussion

We tested the relative extent to which published methods for chemically extracting carotenoid pigments from avian plasma recovered both polar and non-polar carotenoids. A diversity of extraction methods have been used with avian plasma, so it was important at this phase of investigation in the field to compare methods from different labs and determine if there is an optimal procedure or if all are equally effective. We found no statistically significant differences in polar carotenoid (xanthophyll) recovery among the methods used. This result is not wholly surprising, as all methods contained a solvent or mixture in which xanthophylls should be highly miscible (Britton 1985) and as prior comparisons of chemical methods for extracting carotenoids from plant leaves yielded no sig-

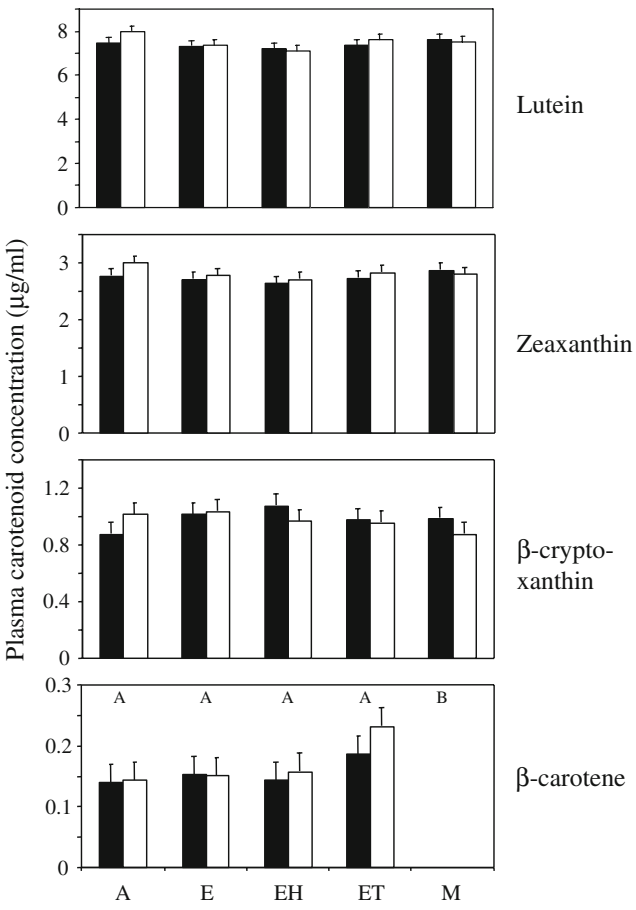

Fig. 2 Bar charts depicting carotenoid recoveries for different solvent extraction methods and centrifuge times in mallard duckling plasma. See Fig. 1 legend for additional details

nificant differences for lutein (Dunn et al. 2004). The similar performance of the procedures, however, is comforting and suggests that prior data collected on polar xanthophylls using any of these five methods should be comparable across studies and species, at least for HPLC-based measurements; we await comparable examinations for those studies that use absorbance spectrophotometry to quantify carotenoid content. This is especially important because lutein and zeaxanthin are the most common and concentrated carotenoids in circulation in birds (reviewed in McGraw 2006a). Further tests are now needed to understand how these methods comparatively recover metabolically derived plasma xanthophylls like anhydrolutein or ketocarotenoids like astaxanthin or canthaxanthin, though we predict a similar outcome as to the one uncovered in this study. We also recognize that the methods tested here still varied in xanthophyll carotenoid recoveries (as is evident from the fact that not all means are identical within a panel in Figs. 1 and 2), but this variability fell within ranges of measurement error (see "Materials and methods").

In contrast to the xanthophylls, recovery of non-polar carotenoids was dependent upon the type of solvent(s) used. The primary methods employed in the literature performed equally well at extracting β-cryptoxanthin and -carotene from finch and mallard plasma, but a relatively recently added method, using methanol alone, proved weak at recovering β-carotene in both bird species. Methanol alone failed to fully recover β-carotene from plant leaves as well (Dunn et al. 2004). Methanol, along with another method (ethanol–hexane), also poorly recovered β-cryptoxanthin in house finches, where it was more common and concentrated than in mallards. Thus, the use of a broad generalized solvent (or a mix of a hydrophilic and hydrophobic solvent) that captures both polar and non-polar carotenoids is recommended over the use of a more aqueous solvent like methanol alone. The same recommendations are made for human plasma and food (Khachik et al. 1992a, b). This recommendation is especially true when nothing is known of the carotenoid content in the focal species and in bird species where these non-polar pigments are key (e.g., β-cryptoxanthin for attaining maximal plumage redness in house finches; McGraw et al. 2006a) or in high concentration (e.g., common moorhen, *Gallinula chloropus*; American coot, *Fulica americana*; lesser black-backed gull, *Larus fuscus*; Surai et al. 2001).

The other main variable tested here was centrifugation time, and we failed to find any significant effect of spinning extracts for 1 vs. 10 min on the recovery of any type of carotenoid in house finch or mallard plasma. Thus, at least in these two species, substantial time can be saved by centrifuging the plasma extract for a shorter amount of time prior to solvent recovery and analysis. The only apparent benefit we can see to retaining a long centrifuge time might be to allow the formation of a smaller, more solid protein pellet at the bottom of tubes, which is harder to disturb during solvent removal and thus less likely to contaminate the extract with flocculant protein.

In conclusion, we have performed the first comparative test of carotenoid extraction methods in birds and found some important differences among them. Future studies might consider adding additional variables (i.e., vortexing times, plasma/solvent volumes) to further understand the optimal methods for recovering polar and non-polar carotenoids from plasma or serum. We also suggest similar studies of procedures that remove carotenoids from tissues (i.e., thermochemical vs. mechanochemical extractions from feathers and bare parts like bill or leg).

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Appendix List of the 98 published studies that we were able to locate that used one of five main chemical methods (acetone, ethanol + hexane, ethanol + TBME, ethanol, and methanol) to extract carotenoids from avian plasma

Citation	Method	Species	Plasma/solvent ratio	Vortexing description	Centrifugation rate
Ruff et al. (1974)	Acetone-only	Chicken (*Gallus gallus domesticus*)	1:9	Not mentioned	1,000×*g* for 10 min
Ruff and Fuller (1975)	Acetone-only	Chicken (*Gallus gallus domesticus*)	Cited Ruff et al. (1974) for method		
Augustine and Thomas (1979)	Acetone-only	Turkey (*Meleagris gallopavo*)	1:9	Not mentioned	Not mentioned
Augustine and Ruff (1983)	Acetone-only	Turkey (*Meleagris gallopavo*)	1:9	Not mentioned	Not mentioned
Allen (1987a)	Acetone-only	Chicken	1:9	Vortexed	1,500×*g* for 10 min

Appendix (continued)

Citation	Method	Species	Plasma/solvent ratio	Vortexing description	Centrifugation rate
Allen (1987b)	Acetone-only	Chicken	Cited Wilson (1956) for method		
Lillehoj and Ruff (1987)	Acetone-only	Chicken	Cited Ruff et al. (1974) for method		
Augustine (1988)	Acetone-only	Turkey	1:9	Twice for 10 s	1,000×*g* for 10 min
Allen (1992a)	Acetone-only	Chicken	1:10	Mixed well	Not mentioned
Allen (1992b)	Acetone-only	Chicken	Cited Allen (1987a) for method		
Allen (1992c)	Acetone-only	Chicken	Cited Allen (1987b) for method		
Conway et al. (1993)	Acetone-only	Chicken	Cited Allen (1987a) for method		
Allen et al. (1996)	Acetone-only	Chicken	1:10	Not mentioned	Not mentioned
Bortolotti et al. (1996)	Acetone-only	American kestrel (*Falco sparverius*) Loggerhead shrike (*Lanius ludovicianus*)	1:10	Not mentioned	1,500×*g* for 10 min
Allen (1997a)	Acetone-only	Chicken	1:10	Not mentioned	Not mentioned
Allen (1997b)	Acetone-only	Chicken	1:10	Not mentioned	Not mentioned
Allen et al. (1997)	Acetone-only	Chicken	Cited Allen (1992a) for method		
Matthews et al. (1997)	Acetone-only	Chicken	1:4	Vortexed	2,800×*g* for 10 min
Allen and Danforth (1998)	Acetone-only	"	Cited Allen et al. (1996) for method		
Negro et al. (1998)	Acetone-only	American kestrel	1:10	Mixed well	1,500×*g* for 10 min
Tella et al. (1998)	Acetone-only	26 bird sp. from Mexico	1:10–1:40	Well mixed	1,500×*g* for 10 min
Gray et al. (1998)	Acetone-only	Chicken	1:4	Vortexed	1,500×*g* for 10 min
Allen (2000)	Acetone-only	Chicken	Cited Allen et al. (1996) for method		
Allen and Fetterer (2000)	Acetone-only	Chicken	Cited Allen et al. (1996) for method		
Bortolotti et al. (2000)	Acetone-only	American kestrel	1:10	Cited Tella et al. (1998) for method	
Fetterer and Allen (2000)	Acetone-only	Chicken	Cited Allen et al. (1996) for method		
Matthews and Southern (2000)	Acetone-only	Chicken	Cited Allen (1987a) and Matthews et al. (1997) for method		
Negro and Garrido-Fernandez (2000)	Acetone-only	White stork (*Ciconia ciconia*)	1:3	Not mentioned	13,000×*g* for 10 min
Negro et al. (2000)	Acetone-only	White stork (*Ciconia ciconia*)	1:3	Not mentioned	13,000×*g* for 10 min
Zhu et al. (2000)	Acetone-only	Chicken	Cited Allen (1997a) for method		
Fernie and Bird (2001)	Acetone-only	American kestrel	Cited Bortolotti et al. (1996) for method		
Negro et al. (2001)	Acetone-only	Red-legged partridge (*Alectoris rufa*)	1:10	Cited Tella et al. (1998) for method	
Allen and Fetterer (2002a)	Acetone-only	Chicken	Cited Allen et al. (1996) for method		
Allen and Fetterer (2002b)	Acetone-only	Chicken	Cited Allen et al. (1996) for method		
Allen (2003)	Acetone-only	Chicken	1:10	Not mentioned	Not mentioned

Appendix (continued)

Citation	Method	Species	Plasma/solvent ratio	Vortexing description	Centrifugation rate
Bortolotti et al. (2003a)	Acetone-only	Red-legged partridge	Cited Bortolotti et al. (1996) and Tella et al. (1998) for method		
Bortolotti et al. (2003b)	Acetone-only	American kestrel	1:10	Not mentioned	1,500×*g* for 10 min
Fetterer et al. (2003)	Acetone-only	Chicken	1:9	Vortexing	1,000×*g* for 10 min
Zhu et al. (2003)	Acetone-only	Chicken	Cited Allen (1997b) for method		
Allen et al. (2004)	Acetone-only	Chicken	1:10	Not mentioned	Not mentioned
Peters et al. (2004)	Acetone-only	Mallard (*Anas platyrhynchos*)	1:3–1:7	Not mentioned	1,500×*g* for 10 min
Tella et al. (2004)	Acetone-only	80 bird sp. from Mexico	1:9	Mixed	10,000 rpm for 10 min
Allen et al. (2005)	Acetone-only	Chicken	Cited Allen et al. (2004) for method		
Blanco et al. (2005)	Acetone-only	Linnet (*Carduelis cannibina*)	1:5	Shaken/sonicated for 1 min	12,000×*g* for 5 min
Figuerola et al. (2005)	Acetone-only	Greylag goose (*Anser anser*)	1:1	Not mentioned	16,249×*g* for 10 min
Peters et al. (2005)	Acetone-only	Mallard	1:3–1:7	Not mentioned	1,500×*g* for 10 min
Tummeleht et al. (2006)	Acetone-only	Great tit (*Parus major*)	1:10	Not mentioned	1,500×*g* for 10 min
Blas et al. (2006)	Acetone-only	Red-legged partridge	Cited Bortolotti et al. (1996) for method		
Yang et al. (2006)	Acetone-only	Chicken	1:1:4	Not mentioned	1,000×*g* for 10 min
Casagrande et al. (2006)	Acetone-only	Eurasian kestrel (*Falco tinnunculus*)	1:40	Not mentioned	14,000×*g* for 5 min
Horak et al. (2006)	Acetone-only	Greenfinch (*Carduelis chloris*)	1:10	Not mentioned	16,800×*g* for 10 min
Aguilera and Amat (2007)	Acetone-only	Greenfinch (*Carduelis chloris*)	1:20	Mixed with a vortex	10,000 rpm for 10 min
Perez-Rodriguez et al. (2007)	Acetone-only	Red-legged partridge	1:10	Vortexed	10,000 rpm for 10 min
Martinez-Padilla et al. (2007)	Acetone-only	Red grouse (*Lagopus lagopus*)	1:10	Vortexed	10,000 rpm for 10 min
Isaksson and Andersson (2008)	Acetone-only	Great tit	1:19	Not mentioned	Not mentioned
Surai and Speake (1998)	Ethanol/H_2O + hexane	Chicken	Not mentioned	Shaken vigorously for 5 min	Not mentioned
Slifka et al. (1999)	Ethanol + hexane	14 sp. of zoo birds	Not mentioned	Not mentioned	Not mentioned
Surai (2000)	Ethanol + hexane	Chicken	1:1:2.5	Shaken vigorously for 5 min	Not mentioned
Surai and Sparks (2001)	Ethanol + hexane	Chicken	1:1:2.5	Shaken vigorously for 5 min	Not mentioned
Surai et al. (2001)	Ethanol + hexane	Chicken	1:1:2	Stirred vigorously on a vortex	2,000 rpm for 5 min
Blount et al. (2002)	Ethanol/H_2O + hexane	Lesser black-backed gull (*Larus fuscus*)	Cited Surai and Speake (1998) for method		
Breithaupt et al. (2003)	Ethanol + hexane	Chicken	1:2:2	Stirred vigorously on a vortex	2,000 rpm × 5 min
Surai et al. (2003)	Ethanol/H_2O + hexane	Chicken	Cited Surai et al. (2001) for method		
Blount et al. (2003a)	Ethanol + hexane	Zebra finch (*Taeniopygia guttata*)	1:2:35	Vortexed	Not mentioned

Appendix (continued)

Citation	Method	Species	Plasma/solvent ratio	Vortexing description	Centrifugation rate
Blount et al. (2003b)	Ethanol + hexane	Zebra finch (*Taeniopygia guttata*)	1:2:35	Vortexed 20 s per solvent	Not mentioned
Horak et al. (2004)	Ethanol + hexane	Great tit	Cited Surai et al. (2001) for method		
Møller et al. (2005)	Ethanol + hexane	Barn swallow (*Hirundo rustica*)	1:2:25	Vortexed	Not mentioned
Ewen et al. (2006a)	Ethanol + hexane	Hihi (*Notiomystis cincta*)	1:2:13.3	Homogenized	Not mentioned
Ewen et al. (2006b)	Ethanol + hexane	Hihi (*Notiomystis cincta*)	1:25:20	Vortexed	Not mentioned
Biard et al. (2006)	Ethanol + hexane	Blue tit (*Cyanistes caeruleus*)	1:2:25	Mixed	Not mentioned
McGraw et al. (2002)	Ethanol + TBME	Zebra finch	1:8:8	Vortexed	3 min (RPMs set)
McGraw et al. (2003a)	Ethanol + TBME	Zebra finch	1:8:4	Vortexed	4 min (RPMs set)
McGraw et al. (2003b)	Ethanol + TBME	Yellow warbler (*Dendroica petechia*) Common yellowthroat (*Geothlypis trichas*)	1:10:10	Vortexed 5 s per solvent	4 min (RPMs set)
McGraw and Ardia (2003)	Ethanol + TBME	Zebra finch	Cited McGraw et al. (2002, 2003a) for method		
McGraw and Ardia (2004)	Ethanol + TBME	Zebra finch	1:7.5:7.5	With each solvent added	3 min (RPMs set)
McGraw and Nogare (2004)	Ethanol + TBME	5 parrot species	1:7.5:7.5	With each solvent added	16,000×*g* for 4 min
McGraw et al. (2004)	Ethanol + TBME	American goldfinch (*Carduelis tristis*) Zebra finch	Cited McGraw et al. (2002) for method		
McGraw and Schuetz (2004)	Ethanol + TBME	3 estrildid finch species	1:10:10	With each solvent added	3 min (RPMs set)
McGraw and Gregory (2004)	Ethanol + TBME	American goldfinch	1:7.5:7.5	5 s with each solvent added	3 min (RPMs set)
McGraw (2004)	Ethanol + TBME	11 songbird species	Cited McGraw et al. (2002) for method		
McGraw et al. (2005)	Ethanol + TBME	American goldfinch	1:7.5:7.5	5 s with each solvent added	3 min (RPMs set)
McGraw and Ardia (2005)	Ethanol + TBME	Zebra finch	Cited McGraw et al. (2002) for method		
McGraw (2005)	Ethanol + TBME	Society finch (*Lonchura domestica*) House finch (*Carpodacus mexicanus*)	1:10:10	Vortexed	10,000 rpm for 4 min
McGraw (2006b)	Ethanol + TBME	Zebra finch	Cited McGraw et al. (2002) for method		
McGraw and Parker (2006)	Ethanol + TBME	Zebra finch	Cited McGraw et al. (2003a) for method		
McGraw and Klasing (2006)	Ethanol + TBME	Red junglefowl (*Gallus gallus*)	Cited McGraw et al. (2002) for method		
McGraw et al. (2006a)	Ethanol + TBME	House finch	Cited McGraw et al. (2002) for method		
McGraw et al. (2006b)	Ethanol + TBME	Zebra finch	Cited McGraw et al. (2003a) for method		
McGraw et al. (2006c)	Ethanol + TBME	Society finch	Cited McGraw (2005) for method		

Appendix (continued)

Citation	Method	Species	Plasma/solvent ratio	Vortexing description	Centrifugation rate
McGraw and Ardia (2007)	Ethanol + TBME	Zebra finch	Cited McGraw et al. (2003a, b) for method		
Ninni et al. (2004)	Ethanol-only	Barn swallow	1:9	Vortexed 1 min	1,500×*g* for 10 min
Alonso-Alvarez et al. (2004)	Ethanol-only	Zebra finch	1:9	Mixed in a vortex	1,500×*g* for 10 min
Costantini and Dell'Omo (2006)	Methanol-only	Eurasian kestrel	1:8	Not mentioned	12,000×*g* for 5 min
Costantini et al. (2006)	Methanol-only	Eurasian kestrel	1:8	Not mentioned	12,000×*g* for 5 min
Costantini et al. (2007a)	Methanol-only	Eurasian kestrel	1:8	Not mentioned	12,000×*g* for 5 min
Costantini et al. (2007b)	Methanol-only	Eurasian kestrel	1:8	Not mentioned	12,000×*g* for 5 min
Costantini et al. (2007c)	Methanol-only	Eurasian kestrel	1:8	Not mentioned	12,000×*g* for 5 min.
Casagrande et al. (2007)	Methanol-only	Eurasian kestrel	1:8	Not mentioned	Not mentioned

Studies using each extraction method are organized in chronological order. Other method parameters, such as plasma/solvent ratio, vortexing, and centrifugation rate, are also reported for comparison and for justification of some of our procedures (see text).

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