Methods for extracting and analyzing carotenoids from bird feathers

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Abstract

Carotenoid pigments serve many endogenous functions in organisms, but some of the more fascinating are the external displays of carotenoids in the colorful red, orange and yellow plumages of birds. Since Darwin, biologists have been curious about the selective advantages (e.g., mate attraction) of having such ornate features, and, more recently, advances in biochemical methods have permitted researchers to explore the composition and characteristics of carotenoid pigments in feathers. Here we review contemporary methods for extracting and analyzing carotenoids in bird feathers, with special attention to the difficulties of removal from the feather keratin

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matrix, the possibility of feather carotenoid esterification and the strengths and challenges of different analytical methods like high-performance liquid chromatography and Raman spectroscopy. We also add an experimental test of current common extraction methods (e.g., mechanical, thermochemical) and find significant differences in the recovery of specific classes of carotenoids, suggesting that no single approach is best for all pigment or feather types.

1. Introduction

Animals utilize a variety of striking features, ranging from elaborate antlers in deer to complex songs and brilliant colors in birds, and these have fascinated biologists for decades (Andersson, 1994). Among the colorful traits expressed by animals, carotenoid-based plumage in birds has garnered special attention. Carotenoids generate an array of colors (e.g., red, orange, yellow) and are often sexually dimorphic, with males typically displaying more elaborate coloration (Blount & McGraw, 2008; McGraw, 2006; McGraw & Blount, 2009). The conspicuousness of these traits and the tractability of carotenoid analysis have led scientists from many disciplines, including behavioral ecology, ecophysiology and evolutionary biology, to focus on these traits as a model system for understanding the mechanisms, functions, evolutionary history and diversification of signaling traits.

There is considerable evidence that the carotenoid-based plumage colors of many bird species are sexually selected traits (Cooney et al., 2019; Delhey & Peters, 2017; Hill, 2006). For example, female house finches (*Haemorhous mexicanus*) prefer to mate with males with the reddest and most saturated ketocarotenoid-based plumage coloration (Giraudeau, Toomey, Hutton, & McGraw, 2018; Hill, 2002; Toomey & McGraw, 2012). A major focus of avian carotenoid research is to investigate why these traits are targets of sexual selection and how carotenoid-based colors have elaborated and diversified. Current hypotheses posit that carotenoid-based plumage colors are signals that transmit reliable information about an individual bird's diet quality, immune and antioxidant function and/or carotenoid processing mechanisms linked to vital cellular processes (Blount & McGraw, 2008; Koch & Hill, 2018; Koch, Josefson, & Hill, 2017; Powers & Hill, 2021; Weaver, Koch, & Hill, 2017; Weaver, Santos, Tucker, Wilson, & Hill, 2018). The analysis of feather carotenoid composition and content is essential to test these hypotheses. The aims of these analyses include: (1) linking feather appearance to the types and amounts of carotenoids present (e.g., Saks, McGraw, & Hõrak, 2003); (2) defining the mechanisms of metabolism

and uptake that underlie plumage color diversity (e.g., Lopes et al., 2016; Toomey et al., 2017, 2018); and (3) tracing the allocation of carotenoid resources to feathers and other internal organs and functions (e.g., Isaksson & Andersson, 2008; McGraw, Nolan, & Crino, 2006; McGraw & Toomey, 2010). The success of these aims requires accurate and precise identification and quantification of feather carotenoids.

Here we review recent literature on the key characteristics and patterns of carotenoid accumulation in bird feathers. We follow this with a summary of the current methodological approaches for extraction, processing and analysis of feather carotenoids. Interestingly, though several methods have been commonly used—namely mechanical grinding (Stradi, Celentano, & Nava, 1995) and thermochemical (Hudon & Brush, 1992) procedures—we know of no quantitative comparisons of these methods and how they perform on different species and feather carotenoid types. Therefore, we include a small-scale experimental test of the impact of popular extraction and processing techniques on the recovery of carotenoids from the red and yellow feathers of commonly studied songbirds (canaries and Gouldian finches) that are pigmented with different types of carotenoids. We find that no single approach is best for all of the carotenoid types we examined and recommend that researchers test multiple extraction and processing methods when initiating studies in a new system.

1.1 Distribution and diversity of feather carotenoids

It is estimated that >29% of all bird species, and 95 out of 236 families, have carotenoid-pigmented plumage (Thomas, McGraw, James, & Madden, 2014). The carotenoid composition of bird feathers has been extensively investigated for nearly a century (Brockmann & Völker, 1934) and >40 carotenoid types have been identified (Table 1; LaFountain, Prum, & Frank, 2015). In studies of songbirds (Order Passeriformes, which comprise half of all bird species), such as widowbirds (*Ploceidae*) and finches (*Fringillidae*), plumage carotenoids have been broadly classified into diet-derived yellow, metabolically modified yellow, or metabolically modified red pigments (Andersson et al., 2007; Ligon et al., 2016). Diet-derived yellow carotenoids (e.g., lutein and zeaxanthin) are common in the foods (e.g., insects, plant parts) of songbird taxa and are deposited unmodified into feathers (Fig. 1). Carotenoids like the canary xanthophylls, 3′-dehydrolutein, astaxanthin and canthaxanthin are classified as metabolically derived because they are not typically found in the diet or circulating

Table 1 Summary of carotenoid pigments characterized from the colorful feathers of different bird species published since McGraw (2006), including extraction and analysis methods and estimates of concentration where available.

Order	Family	Species	Patch	Patch color	Extraction method	Separation and/or detection method[a]	Major carotenoids noted[b]	Concentration of total carotenoids (μg g^{-1} feather)	References
Anseriformes	Anatidae	*Rhodonessa caryophyllacea*	Crown	Pink	No extraction	Raman	15	Not reported	Thomas and James (2016)
Galliformes	Phasianidae	*Chrysolophus pictus*	Mantle	Orange	Acidified pyridine	HPLC, ESI-MS, Raman	1,2, 17	Not reported	Gao, Song, Tong, and Li (2016)
			Crown, Breast	Yellow, Red	Acidified pyridine	HPLC, UV–Vis, Raman	1, 2	Not reported	Gao et al. (2018)
Columbiformes	Columbidae	*Ptilinopus magnificus septentrionalis*	Breast	Burgundy	Acidified pyridine	HPLC, UV–Vis	18	Not reported	Berg, LaFountain, Prum, Frank, and Tauber (2013)
		Ptilinopus pulchellus pulchellus	Breast	Red	Acidified pyridine	HPLC, UV–Vis	18	Not reported	Berg et al. (2013)
		Ptilinopus solomonensis speciosus	Breast	Purple	Acidified pyridine	HPLC, UV–Vis	18	Not reported	Berg et al. (2013)
		Ptilinopus jambu	Breast	Pink	Acidified pyridine	HPLC, UV–Vis	18	Not reported	Berg et al. (2013)
Charadriiformes	Laridae	*Sterna dougallii*	Breast	Pink	Acidified pyridine	TLC, Abs. spec	12	Not reported	Hays, Hudon, Cormons, Dicostanzo, and Lima (2006)
Pelecaniformes	Ardeidae	*Butorides virescens*	Powder Down	Yellow	Acidified pyridine and Raman	HPLC, UV–Vis, Raman	1, 2, 10, 11	Not reported	Thomas and McGraw (2018)
		Eudocimus ruber	Back	Red	Acidified pyridine	HPLC, UV–Vis, APCI MS	15	Not reported	Mendes-Pinto et al. (2012)

Piciformes	Lybiidae	*Pogoniulus pusillus*	Crown	Red	No extraction	Raman	13	Not reported	Kirschel et al. (2020)
		Pogoniulus chrysoconus	Crown	Yellow	No extraction	Raman	1	Not reported	Kirschel et al. (2020)
	Picidae	*Colaptes auratus*	Third Rectrix	Red	Methanol grinding	HPLC, UV–Vis	1,2, 3,10, 12, 13, 14, 15, 16, 17	Not reported	Hudon, Wiebe, and Stradi (2021)
		C. auratus		Red	Pyridine	HPLC, UV–Vis	1, 10, 18	Not reported	Hudon et al. (2016)
Passeriformes	Eurylaimidae	*Cymbirhynchus macrorhynchos*	Breast	Red	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 25	Not reported	Prum, LaFountain, Berg, Tauber, and Frank (2014)
		Eurylaimus ochromalus	Flank	Yellow	Acidified pyridine, saponification	HPLC, UV–Vis, APCI MS	25, 26	Not reported	Prum et al. (2014)
		E. ochromalus	Breast	Rosy-Pink	Acidified pyridine, saponification	HPLC, UV–Vis, APCI MS	25, 26	Not reported	Prum et al. (2014)
		Sarcophanops steerii	Breast	Purple-Maroon	Acidified pyridine	HPLC, UV–Vis, APCI MS	25	Not reported	Prum et al. (2014)
		Eurylaimus javanicus	Breast	Purple-Maroon	Acidified pyridine, saponification	HPLC, UV–Vis, APCI MS	26	Not reported	Prum et al. (2014)
		E. javanicus	Back	Yellow	Acidified pyridine, saponification	HPLC, UV–Vis, APCI MS	25	Not reported	Prum et al. (2014)
		Psarisomus dalhousiae	Throat	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 2	Not reported	Prum et al. (2014)

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		P. dalhousiae	Belly	Green	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 2	Not reported	Prum et al. (2014)
		Calyptomena viridis	Belly	Green	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 27, 28	Not reported	Prum et al. (2014)
	Pipridae	*Ilicura militaris—Red variant*	Forehead, Rump	Red	Methanol grinding	HPLC, UV–Vis	1, 2, 3, 6, 7, 10, 18, 44	Not reported	Hudon, Anciães, Bertacche, and Stradi (2007)
		I. militaris—Yellow variant	Forehead, Rump	Yellow	Methanol grinding	HPLC, UV–Vis	2, 3	Not reported	Hudon, Storni, Pini, Anciães, and Stradi (2012)
		Masius chrysopterus	Crown	Yellow	Methanol grinding	HPLC, UV–Vis	1, 6, 7	Not reported	Hudon et al. (2012)
		Neopelma pallescens	Forehead	Yellow	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7	Not reported	Hudon et al. (2012)
		Machaeropterus regulus	Forehead	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7, 18, 44	Not reported	Hudon et al. (2012)
		Lepidothrix coronata	Breast	Yellow	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7	Not reported	Hudon et al. (2012)
		Lepidothrix natteteri	Breast	Yellow	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7	Not reported	Hudon et al. (2012)

	Lepidothrix serena	Breast	Yellow	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7	Not reported	Hudon et al. (2012)
	Antilophia galeata	Head	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7, 18, 44	Not reported	Hudon et al. (2012)
	Chiroxiphia pareola	Head	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7, 18, 44	Not reported	Hudon et al. (2012)
	Chiroxiphia caudata	Head	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7, 18, 44	Not reported	Hudon et al. (2012)
	Heterocercus linteatus	Crown	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	2, 12, 13, 16	Not reported	Hudon et al. (2012)
	Pipra aureola	Head	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7, 18, 44	Not reported	Hudon et al. (2012)
		Breast	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7, 18, 44	Not reported	Hudon et al. (2012)
		Neck	Yellow	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7, 18	Not reported	Hudon et al. (2012)
	Pipra filicauda	Head	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7, 18, 44	Not reported	Hudon et al. (2012)
		Breast	Yellow	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7	Not reported	Hudon et al. (2012)

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Order	Family	Species	Patch	Patch color	Extraction method	Separation and/or detection method[a]	Major carotenoids noted[b]	Concentration of total carotenoids ($\mu g\ g^{-1}$ feather)	References
		Pipra fasciicauda	Head, Breast	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7, 18, 44	Not reported	Hudon et al. (2012)
		Pipra erythrocephala	Head	Yellow	Methanol grinding	HPLC, UV–Vis, ESI-MS	1, 2, 7	Not reported	Hudon et al. (2012)
		Pipra rubrocapilla	Head	Red	Methanol grinding	HPLC, UV–Vis, ESI-MS	2, 12, 13, 14, 15, 16	Not reported	Hudon et al. (2012)
	Cotingidae	*Xipholena punicea*	Not indicated	Burgundy	Acidified pyridine	HPLC, UV–Vis, APCI MS, ^{1}H NMR	12, 15, 25, 29, 30, 31, 32, 33	Not reported	LaFountain et al. (2010) and LaFountain, Pacheco, Prum, and Frank (2013)
		Phoenicircus carnifex	Body	Red	Acidified pyridine	HPLC, UV–Vis	1, 18	Not reported	Berg et al. (2013)
		Carpornis cucullatus	Belly	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	1	Not reported	Prum, LaFountain, Berro, Stoddard, and Frank (2012)
		Snowornis subalaris	Underwing Coverts	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	1	Not reported	Prum et al. (2012)
		Phibalura flavirostris	Belly	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	1	Not reported	Prum et al. (2012)
		Ampelion rufaxilla	Belly	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	1	Not reported	Prum et al. (2012)

	Ampeliodes tschudii	Undertail Coverts	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	1	Not reported	Prum et al. (2012)
	Tijuca atra	Secondaries	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	1	Not reported	Prum et al. (2012)
	Pipreola aureopectus	Belly	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 2	Not reported	Prum et al. (2012)
	Pipreola formosa	Belly	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 2	Not reported	Prum et al. (2012)
	P. formosa	Throat	Orange	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 2	Not reported	Prum et al. (2012)
	Pipreola chlorolepidota	Throat	Orange	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 2	Not reported	Prum et al. (2012)
	Pipreola whitelyi	Breast	Orange	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 13	Not reported	Prum et al. (2012)
	Procnias tricarunculatus	Belly	Yellow	Acidified pyridine	HPLC, UV–Vis, APCI MS	6, 7	Not reported	Prum et al. (2012)
	Pyroderus scutatus scutatus	Throat	Orange	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 6, 13, 15	Not reported	Prum et al. (2012)
	Pyroderus scutatus occidentalis	Throat	Orange	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 6, 13, 15	Not reported	Prum et al. (2012)

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Order	Family	Species	Patch	Patch color	Extraction method	Separation and/or detection method[a]	Major carotenoids noted[b]	Concentration of total carotenoids (μg g^{-1} feather)	References
		P. carnifex	Belly	Red	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 18	Not reported	Prum et al. (2012)
		Rupicola rupicola	Belly	Orange	Acidified pyridine	HPLC, UV–Vis, APCI MS	1, 6, 13, 15, 31	Not reported	Prum et al. (2012)
		Rupicola peruviana saturata	Belly	Orange	Acidified pyridine	HPLC, UV–Vis, APCI MS	2, 34, 35	Not reported	Prum et al. (2012)
		R. peruviana sanguinolenta	Back, Belly	Red	Acidified pyridine	HPLC, UV–Vis, APCI MS	2, 34, 35	Not reported	Prum et al. (2012)
		X. punicea	Back, Belly	Burgundy	Acidified pyridine	HPLC, UV–Vis, APCI MS	12, 15, 25, 29, 30, 31, 32, 33	Not reported	Prum et al. (2012)
		Xipholena lamellipennis	Back	Black	Acidified pyridine	HPLC, UV–Vis, APCI MS	15, 25, 29, 30, 31	Not reported	Prum et al. (2012)
		Xipholena atropurpurea	Back	Black	Acidified pyridine	HPLC, UV–Vis, APCI MS	15, 25, 29, 30, 32	Not reported	Prum et al. (2012)
		Haematoderus militaris	Back, Belly	Red	Acidified pyridine	HPLC, UV–Vis, APCI MS	18, 25, 29, 30, 31	Not reported	Prum et al. (2012)

		Querula purpurata	Throat	Red	Acidified pyridine	HPLC, UV–Vis, APCI MS	29, 30, 31, 32	Not reported	Prum et al. (2012)
		Cotinga cotinga	Breast	Purple	Acidified pyridine	HPLC, UV–Vis, APCI MS	33	Not reported	Prum et al. (2012)
		Cotinga maculata	Breast	Purple	Acidified pyridine	HPLC, UV–Vis, APCI MS	33	Not reported	Prum et al. (2012)
		Cotinga amabilis	Breast	Purple	Acidified pyridine	HPLC, UV–Vis, APCI MS	33	Not reported	Prum et al. (2012)
		Porphyrolaema porphyrolaema	Throat	Purple	Acidified pyridine	HPLC, UV–Vis, APCI MS	25, 33	Not reported	Prum et al. (2012)
		Lipaugus streptophorus	Undertail Coverts	Rosy-Pink	Acidified pyridine	HPLC, UV–Vis, APCI MS	25, 33	Not reported	Prum et al. (2012)
	Tityridae	*Iodopleura isabellae*	Pectoral Tufts	Purple	Acidified pyridine	HPLC, UV–Vis, APCI MS	15	Not reported	Mendes-Pinto et al. (2012)
	Maluridae	*Malurus melanocephalus*	Back	Red	Methanol grinding	HPLC, UV–Vis	1,2, 6,7,12, 13, 14, 15	1227.91 ± 197.48	Rowe and McGraw (2008)
	Oriolidae	*Oriolus cruentus*	Breast	Red	Acidified pyridine	HPLC, UV–Vis, APCI MS	12, 13, 14, 15, 21	Not reported	LaFountain, Frank, and Prum (2013)
		Oriolus traillii	Breast	Maroon	Acidified pyridine	HPLC, UV–Vis, APCI MS	13, 15, 36	Not reported	LaFountain, Frank, and Prum (2013)
	Malaconotidae	*Laniarius atroflavus*	Breast	Yellow, Orange	Acidified pyridine	HPLC, UV–Vis	6, 7, 37	1162.1	Osinubi et al. (2018)

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	Notiomystidae	*Notiomystis cincta*	Breast	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2, 3, 4	Not reported	Ewen et al. (2006)
	Paridae	*Parus major*	Ventral Plumage	Yellow	Methanol grinding	HPLC, UV–Vis	1, 2	Approx. 40	Isaksson, Ornborg, Prager, and Andersson (2008)
			Ventral Plumage	Yellow	Sonication with *N*,*N*-dimethylformamide	HPLC, UV–Vis	1, 2	63.6	Senar, Negro, Quesada, Ruiz, and Garrido (2008)
	Regulidae	*Regulus satrapa*	Crown	Orange	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 10, 12, 13, 15, 17	Range 1883–2751	Chui, McGraw, and Doucet (2011)
	Ploceidae	*Euplectes macrourus*	Epaulets	Yellow	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 10	341.0 ± 157.0	Andersson, Prager, and Johansson (2007)
		Euplectes axillaris	Epaulets	Orange-Red	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 7, 10, 11	1715.3 ± 569.7	Andersson et al. (2007)
		Euplectes ardens	Collar	Red	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 7, 10, 11, 12, 13, 14, 15, 38	704.4 ± 227.6	Andersson et al. (2007)
		Euplectes afer	Rump	Yellow	Methanol grinding	HPLC, UV–Vis	1,2,10	788.4 ± 29.8	Prager, Johansson, and Andersson (2009)
		Euplectes orix	Breast	Orange-Red	Methanol grinding	HPLC, UV–Vis	1,2,10,12, 13,14,15, 38	881.3 ± 86.2	Prager et al. (2009)

	Quelea quelea	Breast, Crown	Red	Acidified pyridine	HPLC, UV–Vis	12, 13, 14, 15	Not reported	Walsh, Dale, McGraw, Pointer, and Mundy (2012)
	Euplectes aureus	Rump	Yellow	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 7, 10	778.8 ± 545.8	Twyman, Prager, Mundy, and Andersson (2018)
	Euplectes hordeaceus	Rump	Red	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 7, 10, 12, 13, 14, 15, 38	249.8 ± 184.4	Twyman et al. (2018)
	Euplectes nigroventris	Rump	Red	Methanol grinding	HPLC, UV–Vis	1, 2, 10, 12, 13, 14, 15, 38	562.1 ± 83.5	Twyman et al. (2018)
	Foudia madagascariensis	Breast	Red	Methanol grinding	HPLC, UV–Vis	1, 2, 7, 10, 12, 13, 14, 15, 38	593.8 ± 372.1	Twyman et al. (2018)
	Ploceus capensis	Breast	Yellow	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 7, 10	259.5 ± 132.9	Twyman et al. (2018)
	Ploceus melanocephalus	Belly	Yellow	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 7, 10	209.8	Twyman et al. (2018)
	Ploceus subaureus	Breast	Yellow	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 7, 10	253.5 ± 77.8	Twyman et al. (2018)
	Ploceus velatus	Breast	Yellow	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 7, 10	205.8 ± 113.2	Twyman et al. (2018)
	Quelea erythrops	Crown	Red	Methanol grinding	HPLC, UV–Vis	1, 2, 6, 7, 10, 12, 13, 14, 15, 38	280.3 ± 112.2	Twyman et al. (2018)
Estrildidae	*Erythrura gouldiae*	Head	Red	Acidified pyridine	HPLC, UV–Vis	12, 14, 21	Not reported	Toomey et al. (2018)

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	Fringillidae	*Loxia curvirostra*	Breast	Red	Sonication with *N*,*N*-dimethylformamide	HPLC, UV–Vis	16	52.67 ± 61.4	del Val et al. (2009)
			Rump	Red	Methanol grinding	HPLC, UV–Vis	1, 3, 12, 15, 16, 17	105.13 nmol/g	Cantarero et al. (2020)
		Haemorhous mexicanus	Breast	Red	Methanol grinding	HPLC, UV–Vis	1,6,7,10, 12, 13, 14, 15, 16, 17, 19, 20	Approx. 496	McGraw et al. (2006)
			Breast	Red	Methanol grinding	HPLC, UV–Vis	1, 2, 3, 4, 5, 6, 7, 10, 12, 13, 14, 15, 16, 17, 19, 39, 40, 41	Approx. 6476	Potticary, Morrison, and Badyaev (2020)
		Serinus canaria—yellow lipochrome	Breast	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 6, 7, 8	320.3 ± 161.4	Koch, McGraw, and Hill (2016)
		S. canaria—red factor	Breast	Red	Acidified pyridine	HPLC, UV–Vis	1, 6, 7, 8, 17, 15, 13	590.4 ± 296.4	Koch et al. (2016)
		Fringilla montifringilla	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1	Not reported	Ligon, Simpson, Mason, Hill, and McGraw (2016)
		Euphonia minuta	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	Not reported	Ligon et al. (2016)
		Euphonia violacea	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	Not reported	Ligon et al. (2016)
		Euphonia laniirostris	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	Not reported	Ligon et al. (2016)

	Euphonia xanthogaster	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	Not reported	Ligon et al. (2016)
	Euphonia rufiventris	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	Not reported	Ligon et al. (2016)
	Euphonia musica	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	Not reported	Ligon et al. (2016)
	Mycerobas carnipes	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1	Not reported	Ligon et al. (2016)
	Carpodacus sibiricus	Not indicated	Red	Acidified pyridine	HPLC, UV–Vis	12, 14, 16	Not reported	Ligon et al. (2016)
	Carpodacus thura	Not indicated	Red	Acidified pyridine	HPLC, UV–Vis	16, 19	Not reported	Ligon et al. (2016)
	Linurgus olivaceus	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	6, 7, 8, 9	Not reported	Ligon et al. (2016)
	Spinus pinus	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	6, 7, 8, 9	Not reported	Ligon et al. (2016)
	Rhynchostruthus socotranus	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	6, 7	Not reported	Ligon et al. (2016)
	Pyrrhoplectes epauletta	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 10	Not reported	Ligon et al. (2016)
Cardinalidae	*Piranga rubra*	Belly	Red	Acidified pyridine	HPLC, UV–Vis, APCI MS	15	Not reported	Mendes-Pinto et al. (2012)
Thraupidae	*Ramphocelus melanogaster*	Throat	Red	Methanol grinding	HPLC, ESI-MS	2, 12, 14, 15, 34, 42	Not reported	McCoy et al. (2021)
	Ramphocelus carbo	Breast	Red	Methanol grinding	HPLC, ESI-MS	2, 12, 14, 15, 34, 42	Not reported	McCoy et al. (2021)
	Ramphocelus bresilius	Rump	Red	Methanol grinding	HPLC, ESI-MS	2, 12, 14, 15, 34, 42	Not reported	McCoy et al. (2021)

Continued

Table 1 Summary of carotenoid pigments characterized from the colorful feathers of different bird species published since McGraw (2006), including extraction and analysis methods and estimates of concentration where available.—cont'd

Order	Family	Species	Patch	Patch color	Extraction method	Separation and/or detection method[a]	Major carotenoids noted[b]	Concentration of total carotenoids (μg g^{-1} feather)	References
		Ramphocelus dimidiatus	Rump	Red	Methanol grinding	HPLC, ESI-MS	2, 12, 14, 15, 34, 42	Not reported	McCoy et al. (2021)
		Ramphocelus nigrogularis	Rump	Red	Methanol grinding	HPLC, ESI-MS	12, 14, 15, 34, 42	Not reported	McCoy et al. (2021)
		Ramphocelus passerinii costaricensis	Rump	Red	Methanol grinding	HPLC, ESI-MS	2, 12, 14, 15, 34, 42	Not reported	McCoy et al. (2021)
		R. passerinii	Rump	Red	Methanol grinding	HPLC, ESI-MS	2, 3, 12, 14, 15, 34, 42, 43	Not reported	McCoy et al. (2021)
		Ramphocelus flammigerus icteronotus	Rump	Yellow	Methanol grinding	HPLC, ESI-MS	2, 3, 12, 14, 34	Not reported	McCoy et al. (2021)
		Ramphocelus flammigerus	Rump	Red	Methanol grinding	HPLC, ESI-MS	2, 3, 12, 14, 15, 34, 42	Not reported	McCoy et al. (2021)
		Ramphocelus sanguinolentus	Rump	Red	Methanol grinding	HPLC, ESI-MS	2, 12,14, 15, 34, 42	Not reported	McCoy et al. (2021)
	Parulidae	*Setophaga coronata*	Throat	Yellow	Not reported	HPLC, UV–Vis	1, 2, 10	573–2708	Brelsford, Toews, and Irwin (2017)
	Icteridae	*Icterus bullockii*	Tail	Orange	Acidified pyridine	HPLC, UV–Vis	1,2, 15	1.08–4.59	Sparrow et al. (2017)
			Breast	Orange	Acidified pyridine	HPLC, UV–Vis	1, 6, 7, 15, 17	121.7	Friedman, McGraw, and Omland (2014b)
		Icterus cucullatus	Breast	Yellow	Acidified pyridine	HPLC, UV–Vis	1	99.6	Friedman et al. (2014b)
		Icterus dominicensis	Breast	Yellow	Acidified pyridine	HPLC, UV–Vis	1	68.6	Friedman et al. (2014b)

	Icterus galbula	Breast	Orange	Acidified pyridine	HPLC, UV–Vis	1, 6, 7, 15, 17	157.2	Friedman et al. (2014b)
	Icterus graduacauda	Breast	Yellow	Acidified pyridine	HPLC, UV–Vis	1	115.4	Friedman et al. (2014b)
	Icterus gularis	Breast	Orange	Acidified pyridine	HPLC, UV–Vis	1, 2, 6, 7, 15, 16	157.5	Friedman et al. (2014b)
	Icterus croconotus	Breast	Orange	Acidified pyridine	HPLC, UV–Vis	1, 16	76.7	Friedman et al. (2014b)
	Icterus mesomelas	Breast	Yellow	Acidified pyridine	HPLC, UV–Vis	1	60.4	Friedman et al. (2014b)
	Icterus nigrogularis	Breast	Yellow	Acidified pyridine	HPLC, UV–Vis	1	121.8	Friedman et al. (2014b)
	Icterus pectoralis	Breast	Yellow	Acidified pyridine	HPLC, UV–Vis	1	271.3	Friedman et al. (2014b)
	Icterus pustulatus	Breast	Orange	Acidified pyridine	HPLC, UV–Vis	1, 2, 6, 7, 15, 16	196.3	Friedman et al. (2014b)
	Icterus prosthemelas	Breast	Yellow	Acidified pyridine	HPLC, UV–Vis	1	140	Friedman et al. (2014b)
	Sturnella bellicosa	Not indicated	Red	Acidified pyridine	HPLC, UV–Vis	13, 14	14	Friedman, McGraw, and Omland (2014a)
	Sturnella superciliaris	Not indicated	Red	Acidified pyridine	HPLC, UV–Vis	2, 12, 13, 14, 15, 16	97.9	Friedman et al. (2014a)
	Sturnella militaris	Not indicated	Red	Acidified pyridine	HPLC, UV–Vis	2, 12, 13, 14, 15, 16	55.2	Friedman et al. (2014a)
	Sturnella neglecta	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1	5.2	Friedman et al. (2014a)
	Sturnella magna	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1	6.6	Friedman et al. (2014a)
	Xanthocephalus xanthocephalus	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1	15.7	Friedman et al. (2014a)

Continued

Table 1 Summary of carotenoid pigments characterized from the colorful feathers of different bird species published since McGraw (2006), including extraction and analysis methods and estimates of concentration where available.—cont'd

Order	Family	Species	Patch	Patch color	Extraction method	Separation and/or detection method[a]	Major carotenoids noted[b]	Concentration of total carotenoids (μg g^{-1} feather)	References
			Throat	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2, 3, 4	79.00 ± 41.13	Newbrey and Reed (2011)
		Cacicus cela	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1,2	258.5	Friedman et al. (2014a)
		Cacicus uropygialis	Not indicated	Red	Acidified pyridine	HPLC, UV–Vis	1,6,7, 14, 15	218.7	Friedman et al. (2014a)
		Cacicus leucoramphus	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	352.5	Friedman et al. (2014a)
		Cacicus haemorrhous	Not indicated	Red	Acidified pyridine	HPLC, UV–Vis	6, 7, 12, 14, 15	272	Friedman et al. (2014a)
		Cacicus melanicterus	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	225	Friedman et al. (2014a)
		Psarocolius wagleri	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1	53.6	Friedman et al. (2014b)
		Psarocolius montezuma	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	31	Friedman et al. (2014a)
		Cacicus melanicterus	Not indicated	Yellow	Acidified pyridine	HPLC, UV–Vis	1, 2	225	Friedman et al. (2014a)

[a]Separation and analysis methods: HPLC—high performance liquid chromatography; TLC—thin layer chromatography; UV–Vis—ultraviolet and visible light photodiode array detection; Raman—Raman spectroscopy detection; ESI-MS—electrospray ionization and mass spectral detection; APCI-MS—atmospheric-pressure chemical ionization and mass spectral detection; Abs. spec—light absorbance spectroscopy; ^{1}H NMR—proton nuclear magnetic resonance spectroscopy.

[b]Key to carotenoids: 1—lutein; 2—zeaxanthin; 3—β-cryptoxanthin; 4—β-carotene; 5—α-carotene; 6—canary xanthophyll A; 7—canary xanthophyll B; 8—canary xanthophyll C; 9—canary xanthophyll D; 10—3′-dehydrolutein; 11—2′3′-anhydrolutein; 12—astaxanthin; 13—α-doradexanthin; 14—adonirubin; 15—canthaxanthin; 16—3-hydroxy-echinenone; 17—echinenone; 18—rhodoxanthin; 19—4-oxo-rubixanthin; 20—4-oxo-gazaniaxanthin; 21—papilioerythrinone; 22—7,8-dehydrolutein; 23—7,8,7′,8′-tetrahydro-zeaxanthin; 24—7,8-dehydro-β-cryptoxanthin; 25—2,3-didehydro-pompadourin; 26—eurylaimin; 27—7,8-dihydro-zeaxanthin; 28—calyptomenin; 29—brittonxanthin; 30—pompadourin; 31—xipholenin; 32—2,3-didehydro-xipholenin; 33—cotingin; 34—adonixanthin; 35—rupricolin; 36—4-hydroxy-canary xanthophyll A; 37—isoastaxanthin; 38—β-doradexanthin; 39—rubixanthin; 40—gazaniaxanthin; 41—β-isocryptoxanthin; 42—didehydroastaxanthin; 43—apo-8-carotenal; 44—piprixanthin.

Note, studies reporting the presence of carotenoids without further chemical identification/characterization are not included (Plummer, Bearhop, Leech, Chamberlain, & Blount, 2018; Ranasinghe & Seneviratne, 2017; Thomas, McGraw, James, & Madden, 2014).

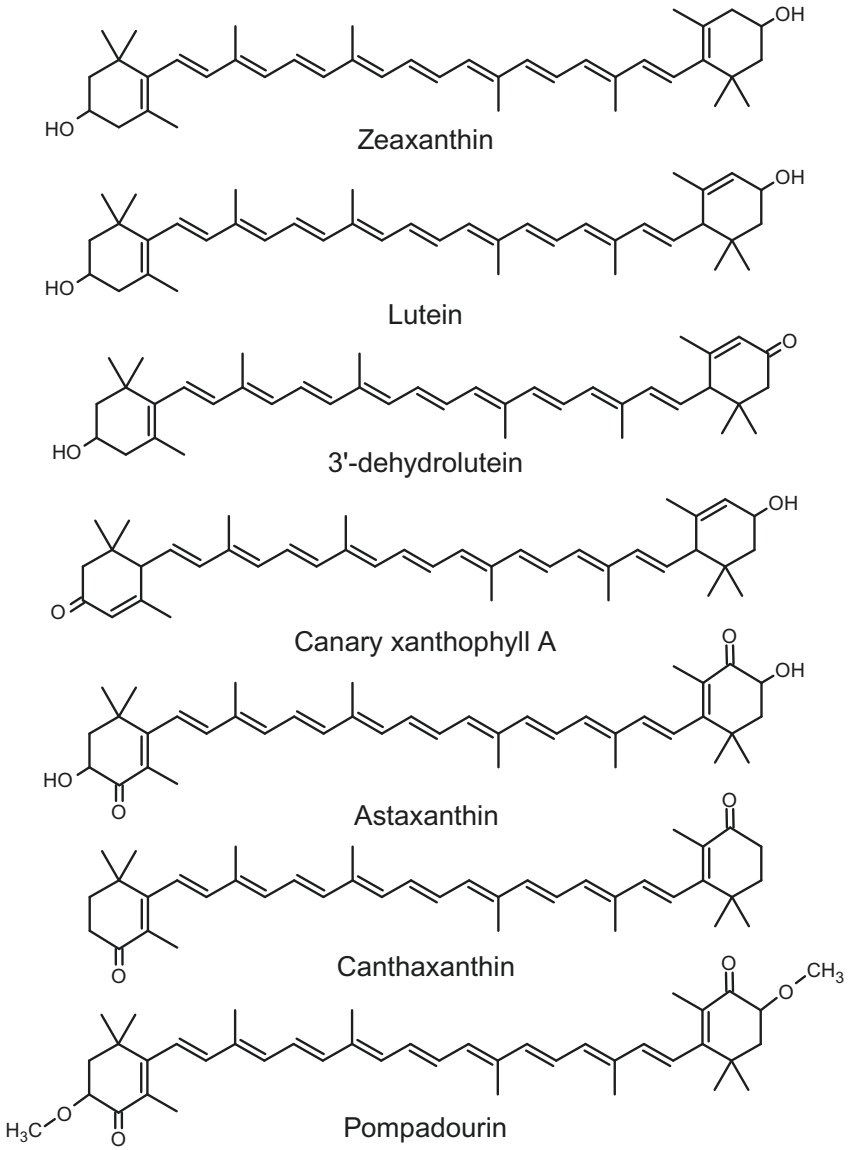

Fig. 1 Structures of a selection of feather carotenoids.

in the plasma of wild birds (McGraw, 2006) and accumulate in the feathers of captive birds with controlled diets that do not contain these pigments (e.g., Koch et al., 2016). Metabolically modified yellow carotenoids (e.g. canary xanthophylls) have chromophores of the same length or shorter than diet-derived yellow carotenoids but their structures differ from their

diet-derived precursors (Fig. 1). Metabolically derived red carotenoids (e.g., astaxanthin, canthaxanthin; Fig. 1) have ketone groups in the C4 and/or C4,4′ position of a β-ring end group that extend the conjugated system, resulting in a bathochromatic shift in the absorbance spectrum that produces red coloration. Genetic evidence from several songbird species and northern flickers (*Colaptes auratus*) indicate that the enzyme cytochrome P450 2J19 mediates the transformation of yellow dietary carotenoid precursors into C4-ketocarotenoids (Aguillon, Walsh, & Lovette, 2021; Kirschel et al., 2020; Lopes et al., 2016; Mundy et al., 2016). Feathers may contain complex mixtures of all three of these carotenoid types and composition can vary widely (Table 1), including among the feather patches of an individual bird, between the sexes and age classes of a given species, and among species. Even species with apparently similar plumage coloration may have distinct feather carotenoid composition (e.g., compare yellow plumage of Gouldian finch and yellow canary in Section 2).

The broad classifications of diet-derived yellow, metabolically modified yellow, or metabolically modified red carotenoids can be useful when investigating questions related to plumage color evolution, diversification and signal information content within specific taxa of birds. However, these classifications are not universal. For example, C4-ketocarotenoids are present in the diets of aquatic and marine birds and can accumulate unmodified in feathers (e.g., Franklin's gull, *Leucophaeus pipixcan,* and roseate tern, *Sterna dougallii*; McGraw & Hardy, 2006; Hays et al., 2006), and "yellow" carotenoids may produce red feather coloration through carotenoid-protein interactions (Stradi, Celentano, Rossi, Rovati, & Pastore, 1995; Veronelli, Zerbi, & Stradi, 1995). Additionally, the palette of carotenoid-based plumage colors extends beyond yellow, orange and red. In the cotingas (*Cotingidae*), methoxy-carotenoids produce striking burgundy and purple plumages, apparently through complex interactions with other components of the feather matrix (LaFountain et al., 2010; Prum et al., 2012; Fig. 2). Discussing the full diversity of plumage carotenoids is beyond the scope of our aims here, but we provide a summary of recent observations (Table 1) and encourage the reader to explore excellent reviews and syntheses of the topic (LaFountain et al., 2015; Morrison & Badyaev, 2016).

The appearance of carotenoid-pigmented plumage not only reflects the carotenoid types in the feather, but also the concentration of pigment. The concentrations of carotenoids in feathers vary widely, with totals ranging from <1 microgram to >6 milligrams per gram of feather (Table 1).

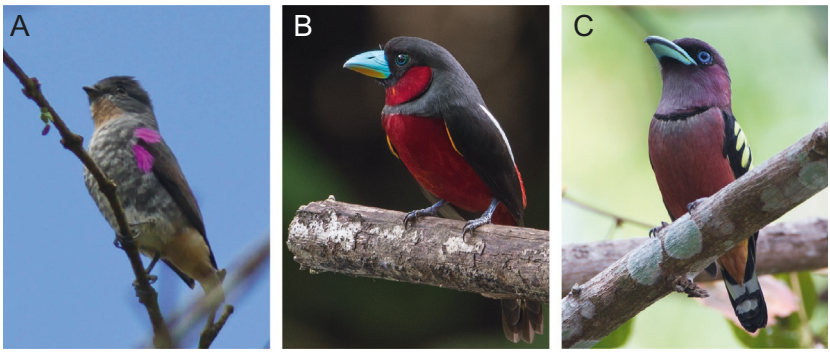

Fig. 2 Representative images of carotenoid-based plumage color diversity. (A) Purple breast feathers from a buff-throated purpletuft (*Iodopleura pipra*; credit: Creative Commons 3.0); (B) crimson breast feathers from a black-and-red broadbill (*Cymbirhynchus macrorhynchos*; credit: Creative Commons 2.0); and (C) crimson ventral plumage of a banded broadbill (*Eurylaimus javanicus*; credit: Creative Commons 3.0).

This large variation within and among species—ranging from dilute washes of pink carotenoid-based colors in some white gull/tern feathers (McGraw & Hardy, 2006) and co-deposition of small amounts of carotenoids with dark melanins (Hofmann, McGraw, Cronin, & Omland, 2007) to intense pigmentation in small feathers, like the red feathers of house finches (Potticary et al., 2020)—is one reason why colorful bird plumage has become an important model system for the study of carotenoids at many levels of analysis. Measurement of feather carotenoid content and composition offers an opportunity to quantitatively link trait expression to ecology, physiology, genetic variation and evolutionary history. The precise and repeatable quantification of plumage carotenoids is essential to making these connections and drawing robust inferences.

1.2 Chromatographic analyses of feather carotenoids

High-performance liquid chromatography (HPLC) is by far the most popular current approach to identifying and quantifying feather carotenoids (Table 1). HPLC analyses require the extraction and isolation of carotenoids from feather tissue, which is further detailed in Section 1.3. Typical HPLC methods utilize reverse-phase chromatography with C-18 or C-30 stationary phases and a variety of mobile phase compositions and gradients. Separation conditions depend on the complexity of the carotenoid compositions in the feathers, but it is notable that carotenes are rarely encountered in feather extracts (McGraw, 2006) and method development

can typically be focused on optimizing the separation of more polar species. A comprehensive discussion of separation conditions for carotenoids can be found in Britton, Liaaen-Jensen, and Pfander (1994) and Chapter 1 of this volume (Britton, 2022). The majority of HPLC studies of feather carotenoids use a UV–Vis diode array detector as the primary means of detection (Table 1), although several studies have also coupled mass spectral (MS) analysis to HPLC separation to characterize novel feather carotenoids (LaFountain, Frank, & Prum, 2013; LaFountain, Pacheco, et al., 2013; McCoy et al., 2021; Prum et al., 2014). MS analyses of feather carotenoids have used a range of ionization methods including atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), and the efficiency of these approaches are known to vary among carotenoid types (Rivera, Vilaró, & Canela, 2011). Therefore, identification and quantification are best achieved through an approach that includes both UV–Vis and MS detection modalities.

1.3 Feather carotenoid extraction

A major challenge in studying feather carotenoids is the effective extraction of the pigments from the feather matrix (Hudon & Brush, 1992). Extraction requires balancing harsh techniques required to disrupt or penetrate the tough feather tissue with the tendency of carotenoids to degrade under such conditions. Mature feathers are composed primarily of keratin proteins with associated lipids and, given their functions in flight and protection, feathers are typically strong, stable and resistant to mechanical disruption (Alibardi, 2017; Terrill & Shultz, 2021). Precisely how carotenoid pigments are integrated in the feather matrix is not fully understood, though evidence suggests that carotenoids may be associated with lipid globules (Lucas & Stettenheim, 1972; Menon & Menon, 2000) and/or bound to proteins (Mendes-Pinto et al., 2012). In our survey of recent literature (Table 1), two methods are primarily used in feather carotenoid extraction: (1) mechanical disruption; and (2) thermochemical treatment, with the former used most often. Mechanical disruption typically involves using a grinding mill to grind feather barbs in solvent (e.g., methanol) with a zirconium oxide ball and jar (e.g., Retsch MM2000) followed by centrifugation/filtration of the homogenate (e.g., Andersson et al., 2007). Thermochemical extraction typically involves the incubation of colorful feather portions in acidified pyridine at 95 °C for 3 h followed by the addition of water and extraction with hexane:*tert*-butyl methyl ether (1:1, v:v) (McGraw, Hudon, Hill, & Parker, 2005). Despite the wide application of these two extraction

methods, we do not know of any studies comparing these approaches or validating their efficiency and effectiveness. It is well known that different types of carotenoids can have different solubility in a given solvent and vary in their susceptibility to oxidation and degradation (Boon, McClements, Weiss, & Decker, 2010; Britton et al., 1994). Therefore, we wondered if and how mechanical and thermochemical extraction techniques vary in their recoveries of different carotenoid types from feather tissue; we address this question in a new study presented in Section 2.

1.4 Saponification of carotenoid esters

Esterified carotenoids are a major component of avian tissues like the retina (Toomey & McGraw, 2007) and skin (García-de Blas, Mateo, Viñuela, Pérez-Rodríguez, & Alonso-Alvarez, 2013; Pérez-Rodríguez, de Blas, Martínez-Padilla, Mougeot, & Mateo, 2016). The esterification of carotenoids dramatically alters their chromatographic behavior, making identification and quantification difficult because authentic standards of carotenoid esters are not generally available. Carotenoid esters have only occasionally been reported in feathers and do not appear to be a major component of the feather carotenoid profile. However, Brush (1967) noted that esterified carotenoids were abundant in the recently molted feathers of male and female scarlet tanagers (*Piranga olivacea*) in the autumn, but absent in feathers collected in other seasons. Brush's (1967) account is consistent with our own observations (M. Toomey and K. McGraw, unpublished) in another songbird species, the house finch, and suggests that esterification may play a role in the initial development of feather pigmentation, but that these ester groups are lost or degraded as the mature feathers age. As with other tissues, alkaline saponification methods have been applied to feather extracts to remove esters and prepare samples for chromatographic analyses (Brush, 1967; Gazda, Toomey, et al., 2020; Prum et al., 2014). However, concerns have been raised that alkaline saponification leads to the selective degradation of carotenoids with an ε-ring and C3 carbonyl groups (e.g., canary xanthophylls) (Gazda, Toomey, et al., 2020; Prum et al., 2014). In Section 2, we address this concern and examine the stability of canary xanthophylls and other classes of feather carotenoids under typical alkali saponification conditions.

1.5 Non-destructive means of feather carotenoid analysis

A limitation of most traditional methods for analyzing plumage carotenoids has been that the material is destroyed (pulverized) or significantly altered

(decolorized) in the process of carotenoid extraction. This limitation presents a challenge when the feathers of interest are rare or irreplaceable (e.g., from endangered animals, museum skins, or fossils; Burns, McGraw, Shultz, Stoddard, & Thomas, 2017; Thomas, McGraw, James, & Madden, 2014). Thus, researchers have recently explored the utility of non-destructive methods of feather carotenoid analysis, most of which have centered on using either reflectance spectrometry (Toral, Figuerola, & Negro, 2008) or Raman spectroscopy (Thomas, McGraw, Butler, et al., 2014; Veronelli et al., 1995). Reflectance spectrometry has long been employed to objectively quantify light-reflectance characteristics of surfaces, including in colorful animals like birds (Brush, 1990), and because of the unique absorbance/reflectance characteristics of carotenoid-containing feathers (compared to other animal pigments like melanins). Analytical methods (including statistical approaches like discriminant function analysis) have been developed to distinguish carotenoids with this technique (Toral et al., 2008). However, this coarse approach generally will not yield data on feather carotenoid composition (e.g., concentration, proportions of different types), unless a feather or patch contains a single, or dominant, type of carotenoid (e.g., McGraw, Beebee, Hill, & Parker, 2003).

Raman spectroscopy, in contrast, uses laser technology to examine photon scattering and vibrational characteristics (e.g., energy states) in a sample; carotenoids can be identified (i.e., light wavelengths absorbed, atomic vibrational strength) based on their conjugated double-bond backbone, but also can be distinguished from each other (e.g., xanthophylls vs. carotenes) due to their different backbone lengths and functional groups. Veronelli et al. (1995) first deployed Raman methods to differentiate psittacofulvins (a unique class of polyenes) from carotenoids in parrot feathers. Since then, this technique has been widely applied to identify feather carotenoids in many avian clades (Thomas, McGraw, James, & Madden, 2014). Resonance Raman techniques also can yield key insights into the interactions between carotenoids and the feather protein matrix in which the carotenoids are bound, which has important implications for feather coloration. For example, carotenoids can be uniquely twisted or aggregated when protein-bound (LaFountain et al., 2015) and this interaction can lead to novel plumage colors, as in the violet-purple feathers of the white-browed purpletuft (*Iodopleura isabellae*). The purpletuft's feathers contain canthaxanthin, which normally confers an orange-red hue were it not for the unique 'head-to-tail' alignment of the protein-bound canthaxanthin revealed by Raman methods (Mendes-Pinto et al., 2012; Fig. 2).

Prum et al. (2014) also used Raman to confirm the presence of a novel carotenoid, 2,3-didehydro-papilioerythrinone, in the crimson-colored plumage of some broadbill species (Fig. 2). Despite these important characterizations, Raman methods have limited quantitative capabilities and thus should be coupled with other methods in studies where knowledge of carotenoid amounts/levels are paramount.

2. Experimental comparison of common feather-carotenoid extraction and saponification methods

The majority of recent feather carotenoid analyses employ either thermochemical extraction with pyridine or mechanical grinding with methanol. Although these methods are widely applied, we do not know of any studies that have directly compared their recoveries of various carotenoid types from different species. Therefore, we aimed to test if and how the extraction and recovery of diet-derived yellow, metabolically modified yellow and metabolically modified red plumage carotenoids might differ between these methods. Additionally, we investigated the impact of alkaline saponification on these major classes of plumage carotenoids. Note that our methods here are not prescriptive; rather our goal was to compare commonly used methods and highlight factors that researchers should consider and validate for their system when planning feather carotenoid analysis.

2.1 Feather samples

We collected 200 breast feathers from each of 3 individual Gouldian finches (*Erythrura gouldiae*), yellow lipochrome canaries (*Serinus canaria*) and red-factor canaries (*S. canaria*; Fig. 3). These domesticated birds were originally collected as part of previous studies of the genetic and molecular mechanisms of feather coloration (Lopes et al., 2016; Toomey et al., 2018). We clipped the carotenoid-pigmented barbs from distal portions of each feather and created a large pool of material from each species/breed. We then divided each pool into 12 subsamples. We washed the subsamples three times each in 1 mL of 100% ethanol and then three times in 1 mL of 100% hexane. We air-dried the samples overnight and then measured the mass of each to the nearest 0.01 mg in triplicate with an analytical balance. We used the mean of the triplicate mass measurements for subsequent analyses.

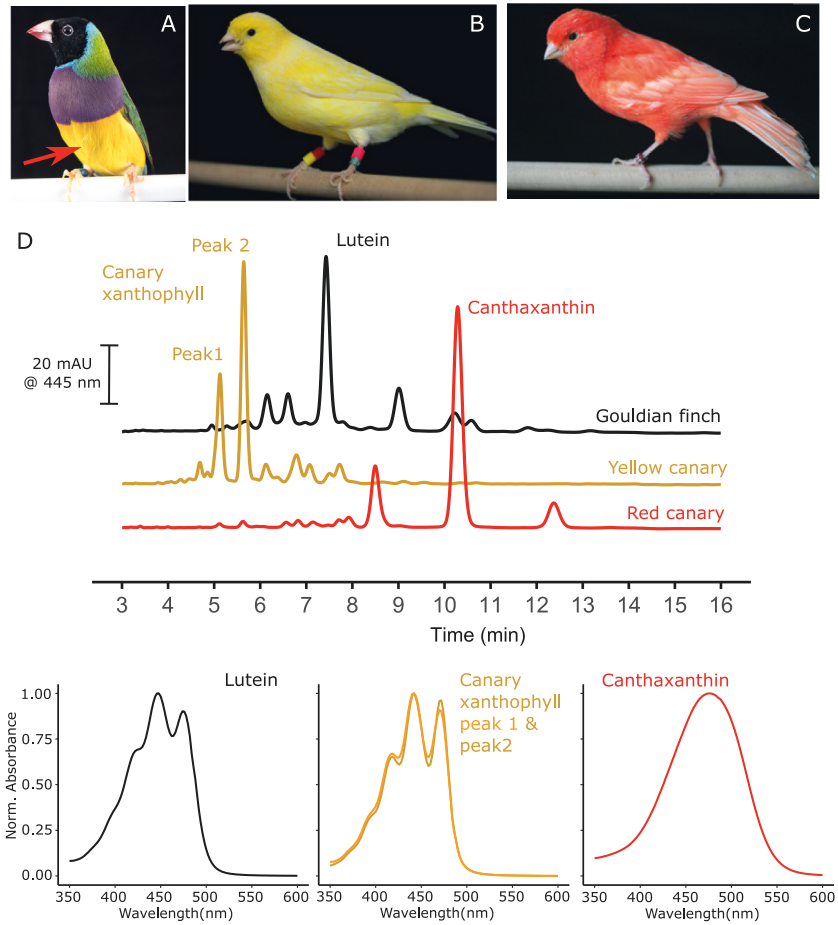

Fig. 3 Representative HPLC chromatograms and UV–Vis absorbance spectra (D) of the major carotenoids in the breast feathers of (A) Gouldian finch, (B) yellow lipochrome canary, and (C) red factor canary. Feather carotenoids in this example were extracted by mechanical disruption (Section 2.2.2). *Bird photos (A–C) are adapted from Toomey, M. B., Marques, C. I., Andrade, P., Araújo, P. M., Sabatino, S., Gazda, M. A., et al. (2018). A non-coding region near Follistatin controls head colour polymorphism in the Gouldian finch. , 285(1888), 20181788 and Lopes, R. J., Johnson, J. D., Toomey, M. B., Ferreira, M. S., Araujo, P. M., Melo-Ferreira, J., et al. (2016). Genetic basis for red coloration in birds. Current Biology 26(11), 1427–1434. Photo credit for (B and C): R. Koch.*

2.2 Extraction methods

We tested three extraction methods that reflect the most common techniques currently in use. We applied each method to four feather subsamples from each of the species/breed. Throughout, we limited light exposure when possible and stored feather extracts, between processing and analysis steps, under nitrogen at −80 °C in the dark. To ensure safety, we carried out all steps

involving solvents and acids in a certified fume hood with the personal protective equipment prescribed in the chemical safety data sheets.

2.2.1 Materials and instrumentation

1. Methanol (Fisher Inc. HPLC Grade, A452, 99.9%)
2. Pyridine (Fisher Inc. ACS Grade, P368, 99%)
3. 12 N Hydrochloric Acid (Fisher Inc. ACS Grade, A144, 37%)
4. Hexane (Fisher Inc. ACS Grade, H292, 98.5%)
5. *tert*-butyl methyl ether (Arcos Organics Inc. 177040025, 99%)
6. Deionized Water
7. 2 mL polypropylene screw cap vial (Prima Inc. PR-SCMC)
8. 2.0 mm zirconia beads (Next Advance, Inc., ZROB20)
9. 9 mL borosilicate glass screw cap tubes (Fisher Inc. 14-959-35C)
10. Beadbug™ homogenizer (Benchmark Sci., D1030)
11. Centrifuge
12. Nitrogen evaporator (Organimation Inc., N-EVP-111)

2.2.2 Methanol grinding

We placed each of four feather-barb subsamples from each species/breed in a 2 mL polypropylene screw cap vial, added 1 mL of methanol and 0.1 g of zirconia beads (2.0 mm, Next Advance, Inc.). We ground the barbs at 4000 Hz for 15 min on a Beadbug™ homogenizer, then centrifuged the sample at 12,000 rcf for 5 min and collected the supernatant into a new sample tube. We then added another 1 mL of methanol to the feather barb sample and repeated grinding, centrifuging and supernatant collection a total of four times until remaining feather material appeared white. We combined the repeated extractions and dried them under a stream of nitrogen.

2.2.3 Acidified pyridine extraction

Following McGraw et al. (2005), we prepared acidified pyridine by adding one drop of 12 N HCl to 25 mL of pyridine. We placed each of four feather barb subsamples from each species/breed in a 9 mL borosilicate glass screw cap tube and added 1 mL of acidified pyridine and 0.1 g of zirconia beads to serve as boiling chips. We then filled the headspace of the tube with nitrogen and capped and incubated the tubes for 3 h at 95 °C. We cooled the tubes to room temperature overnight (16 h), then added 2 mL of distilled deionized water followed by 2 mL of hexane:*tert*-butyl methyl ether (1:1, v:v), and mixed the samples by inverting the tubes for 30 s. We then centrifuged the tubes for 5 min at 2000 rcf, then collected the upper solvent fraction to a new tube and dried the sample under a stream of nitrogen.

2.2.4 Non-acidified pyridine

Acidic conditions may lead to selective degradation of different carotenoid types (Mortensen & Skibsted, 2000). Therefore, we sought to determine if and how removing the acid component from the pyridine extraction affected carotenoid recovery. Methods here were as above for acidified pyridine (Section 2.2.3), but we did not add HCl to the pyridine solvent.

2.3 Saponification

2.3.1 Materials and instrumentation

1. 0.2 M sodium hydroxide (Sigma Inc. ACS Grade, S-0899) dissolved in 100% methanol
2. 0.02 M sodium hydroxide (Sigma Inc. ACS Grade, S-0899) dissolved in 100% methanol
3. Saturated sodium chloride in deionized water
4. Deionized water
5. Hexane (Fisher Inc. ACS Grade, H292, 98.5%)
6. *tert*-Butyl methyl ether (Arcos Organics Inc. 177040025, 99%)
7. 9 mL borosilicate glass screw cap tubes (Fisher Inc. 14–959-35C)
8. 2 mL glass vial (Alwsci Tech., C0001296)
9. Centrifuge
10. Nitrogen evaporator (Organimation Inc., N-EVP-111)

2.3.2 Methods

To test the impact of alkaline saponification on feather carotenoid recovery, we split each of the methanol extracts described above into three aliquots. One aliquot was stored for later analysis without saponification at −80 °C, in the dark, in a 2 mL glass vial, capped under nitrogen. We saponified one aliquot with 1 mL of strong base (0.2 M methanolic NaOH) and the other aliquot with 1 mL of weak base (0.02 M methanolic NaOH). Both treatments were capped under nitrogen and incubated for 4 h at room temperature in the dark. We re-extracted the samples by adding 1 mL of saturated NaCl in water, 2 mL of distilled deionized water and 2 mL of hexane:*tert*-butyl methyl ether (1:1, v:v) and mixed the samples by inverting the tubes for 30 s. We then centrifuged the tubes for 5 min at 2000 rcf, then transferred the upper solvent fraction to a 2 mL glass vial and dried the sample under a stream of nitrogen.

2.4 HPLC analyses

2.4.1 Materials and instrumentation

1. Acetonitrile (Alfa Aesar, HPLC Grade, UN1648, 99.7%)
2. Methanol (Fisher Inc. HPLC Grade, A452, 99.9%)

3. Dichloromethane (Alfa Aesar, HPLC Grade, UN1593, 99.7%)
4. Zeaxanthin (gift of DSM Inc.)
5. Canthaxanthin (gift of DSM Inc.)
6. Agilent 1200 series HPLC
   a. Solvent degasser (G1322A)
   b. Autosampler (G7129A)
   c. Binary pump (G1312B)
   d. Heated column compartment (G1316A)
   e. UV–Vis diode array detector (G7115A)
7. YMC C30 carotenoid column (5 μm, 4.6 mm × 250 mm, YMC Inc., CT99S05-2546WT)

2.4.2 Methods

We characterized carotenoid content of each subsample with HPLC following previously described methods (Gazda, Toomey, et al., 2020). Briefly, we resuspended dried extracts in 200 μL mobile phase solvent (methanol:acetonitrile 1:1 v:v) and injected 50 μL samples into an Agilent 1200 series HPLC with a solvent degasser and UV–Vis diode array detector. We fitted the HPLC with a YMC C30 carotenoid column (5 μm, 4.6 mm × 250 mm, YMC Inc.). We used a gradient mobile phase of acetonitrile:methanol:dichloromethane (44:44:12) (vol:vol:vol) for 0–11 min transitioning to acetonitrile:methanol:dichloromethane (35:35:30) from 11 to 21 min and returning to isocratic conditions through 35 min. We held the column at 30 °C and pumped the mobile phase at a constant rate of 1.2 mL min^{-1}. We monitored the samples with a UV–Vis photodiode array detector at 480 and 445 nm and quantified canthaxanthin with an external standard curve for canthaxanthin. We quantified lutein and the canary xanthophylls relative to an external standard curve for zeaxanthin. The standard curves were calculated over a range from 0.0004 to 0.2 μg.

2.5 Results of feather extraction and saponification methods tests

As expected, the breast feathers of Gouldian finches, yellow lipochrome canaries and red-factor canaries had distinct carotenoid profiles dominated by lutein, canary xanthophylls and canthaxanthin respectively (Fig. 3). To investigate the impacts of extraction and saponification methods, we chose to focus our analyses on these major carotenoid types. We found that extraction method had no significant impact on the recovery of lutein from Gouldian finch feathers ($F_{2,9} = 0.571$, $P = 0.584$; Fig. 4) or canary xanthophylls from the yellow lipochrome canary ($F_{2,9} = 0.982$, $P = 0.411$; Fig. 4). In contrast, the recovery of canthaxanthin from red-factor canary feathers

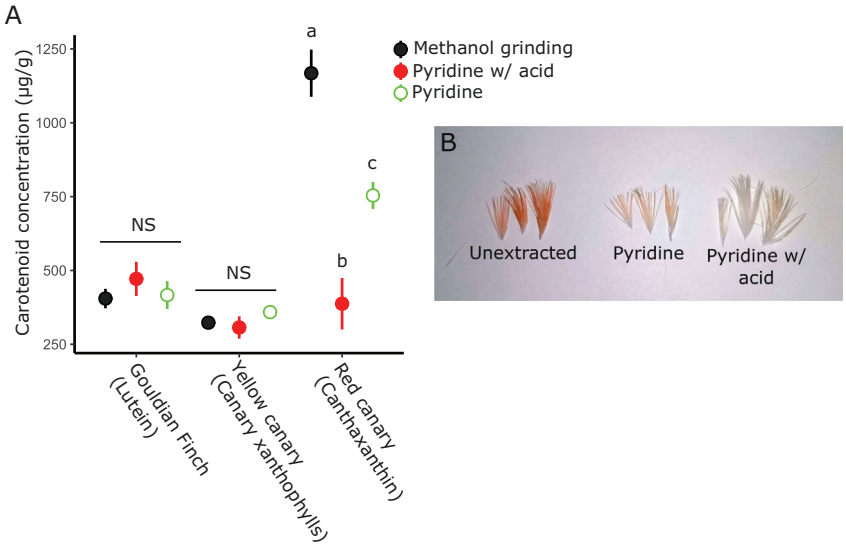

Fig. 4 (A) Mean ∓ s.d. carotenoid mass recovered per gram of colored feather barb using mechanical disruption (methanol grinding), thermochemical extraction with acidification (pyridine with acid), or thermochemical extraction without acidification (pyridine). Treatments labeled with separate letters differed significantly in carotenoid recovery (Tukey's post-hoc test, $P \leq 0.016$). NS indicates no significant differences among treatments. (B) Examples of unextracted, pyridine-extracted and pyridine-with-acid extracted red canary feather barbs.

differed significantly with extraction method ($F_{2,9} = 28.44$, $P = 0.00013$; Fig. 4). Grinding with methanol provided the greatest recovery of canthaxanthin, while we recovered surprisingly small amounts with pyridine extraction, especially the acidified condition (Fig. 4A). Reduced recovery may be attributable to incomplete extraction. The feather barbs in the pyridine condition retained coloration after extraction (Fig. 4B). However, feathers extracted with acidified pyridine retained qualitatively less color (Fig. 4B), suggesting that a process other than incomplete extraction, possibly selective degradation, was responsible for the poor recovery in this condition.

Alkaline saponification had no significant effect on the yields of lutein from Gouldian finch feathers ($F_{2,9} = 0.123$, $P = 0.886$; Fig. 5) or canthaxanthin from red-factor canaries ($F_{2,9} = 1.401$, $P = 0.295$; Fig. 5). However, saponification significantly and dramatically affected the recovery of canary xanthophylls from yellow lipochrome canary feathers ($F_{2,9} = 126.4$, $P = 2.6 \times 10^{-7}$, Fig. 5). Canary xanthophylls were nearly eliminated from samples treated with strong base (Figs. 5 and 6).

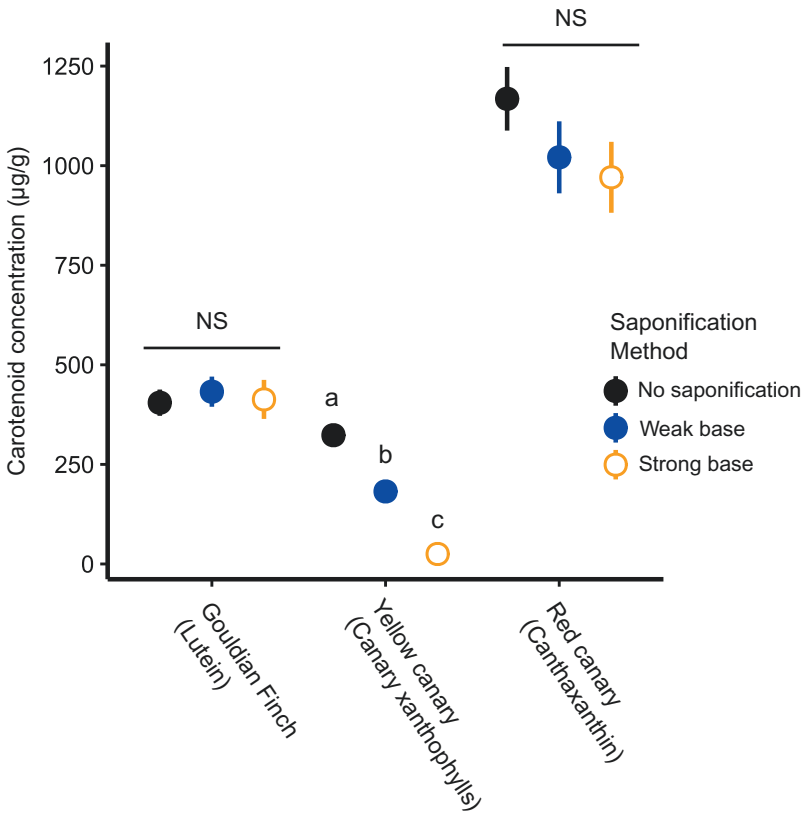

Fig. 5 Mean ∓ s.d. carotenoid mass recovered per gram of feather barbs without saponification (No saponification), with a 0.02 M NaOH treatment (Weak base), or a 0.2 M NaOH treatment (Strong base). Treatments labeled with separate letters differed significantly in carotenoid recovery (Tukey's post-hoc test, $P \leq 4.1 \times 10^{-5}$). NS indicates no significant differences among treatments.

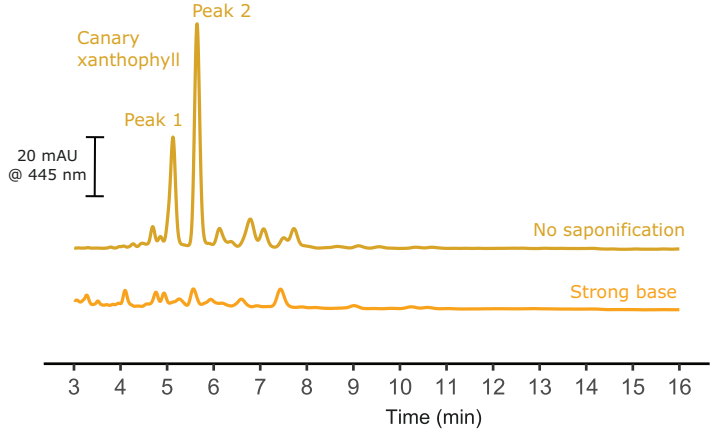

Fig. 6 Representative chromatograms of yellow canary feather extracts without saponification or following a 4-h saponification with 0.2 M NaOH in methanol (Strong base).

3. Recommendations for feather extraction, processing and analysis

Our analyses indicate that the recovery and/or stability of feather carotenoids varies among extraction methods and with carotenoid type. Mechanical disruption in methanol yielded good recoveries of all carotenoid types. Thermochemical extraction with pyridine yielded recoveries of lutein from Gouldian finch feathers and canary xanthophylls from yellow canary feathers that were comparable with mechanical disruption using methanol. In contrast, pyridine extraction of red canary feathers yielded significantly lower recoveries of the C4-ketocarotenoid canthaxanthin when compared to mechanical disruption with methanol. We observed the lowest yields of canthaxanthin in the relatively harsh acidified pyridine condition, suggesting that selective degradation, rather than incomplete extraction, was the cause of the lower yields.

Saponification of the extracts did not improve the yield of free carotenoids from Gouldian finch or red canary feathers and resulted in significant degradation and loss of canary xanthophylls from the yellow canary feather extracts. All of the feather samples in our analysis were collected from captive, domesticated birds, well outside of the molt period. However, as we have noted above, the presence and abundance of esterified carotenoids in feathers may vary with the age of the feathers and be most abundant in recently grown feathers during or shortly after the molt period (Brush, 1967). Additional investigations are needed to assess the time course and abundance of carotenoid esters in feathers, which may yield insights into the mechanisms controlling the development and stability of feather color. Canary xanthophylls have an epsilon end ring and C3 or C3,3′ carbonyl groups, and their selective degradation under alkaline conditions is consistent with observed sensitivity of other carotenoids with a similar configuration (Britton, Liaaen-Jensen, & Pfander, 2004; Buchecker & Eugster, 1979; Prum et al., 2014). Why and how the epsilon C3 carbonyl carotenoids are degraded under alkaline conditions has not been empirically determined.

We recommend that, when initiating a project with a bird species for which feather carotenoid content has not been previously characterized, investigators should pilot test different methods to determine the full complement of carotenoids present, recognize any particular pigment omissions/degradations from those pilot tests, and then pick the method (or methods)

that is/are optimal for recovery of those carotenoids. Our results suggest that both mechanical disruption and thermochemical treatment are effective means of extraction, but that C4-ketocarotenoids may be susceptible to degradation during thermochemical extraction. Therefore, researchers should consider avoiding high temperatures and acidic conditions when working with C4-ketocarotenoids. Similarly, saponification with an alkaline reagent selectively degraded canary xanthophylls. Therefore, analyses involving saponification may lead to an underestimate of the abundance of canary xanthophylls and related pigments in a sample. However, saponification (which entails alkaline treatment) may only be needed for samples from certain species sampled at specific periods during the lifetime of a feather.

4. Conclusions

In recent decades, our understanding of the diversity, mechanisms and functions of carotenoid-based plumage coloration has grown tremendously. Elegant analytical work has identified new carotenoid types and expanded the palette of known carotenoid colors (LaFountain et al., 2010; LaFountain, Frank, & Prum, 2013). The blossoming of avian genomics is revealing the genetic and biochemical mechanisms underlying the rich diversity of plumage carotenoids and colors (Gazda, Araújo, et al., 2020; Gazda, Toomey, et al., 2020; Lopes et al., 2016; Mundy et al., 2016; Toomey et al., 2017). Plumage carotenoids are proving to be a powerful system to connect evolutionary change at the genomic and phenotypic levels (Aguillon et al., 2021; Price-Waldman & Stoddard, 2021; Toews et al., 2016). At the ecosystem level, carotenoid-based plumage color expression can be affected by several environmental and physiological stressors, including rapid anthropogenic change (Hutton & McGraw, 2016; Leveau, 2021). The environmental sensitivity of carotenoid accumulation in bird feathers offers unique opportunities to track environmental quality and examine the evolution of signaling in a dynamic environment. These are just a few examples of the diverse and exciting frontiers of avian plumage carotenoid research, and this progress is built upon a foundation of accurate, precise and repeatable carotenoid analyses.

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