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Xanthophylls in Commercial Egg Yolks: Quantification and Identification by HPLC and LC-(APCI)MS Using a C30 Phase

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The xanthophylls lutein and zeaxanthin have attracted a lot of interest since it was presumed that an increased nutritional uptake may prevent adult macula degeneration (AMD). Although egg yolks serve as an important dietary source of lutein and zeaxanthin, data on xanthophyll concentrations in commercial egg yolks are not available. Thus, an high-performance liquid chromatography−diode array detector (HPLC-DAD) method was developed allowing for simultaneous separation of eight xanthophylls used to fortify poultry feed. Peak identification was carried out by liquid chromatography−atmospheric pressure chemical ionization mass spectrometry [LC-(APCI)MS]. Egg yolks of four types of husbandry (seven batches each) were examined. Lutein and zeaxanthin were the predominant xanthophylls in egg yolks produced in accordance with ecological husbandry (class 0) because the concentrations of these xanthophylls ranged from 1274 to 2478 μg/100 g and from 775 to 1288 μg/100 g, respectively. Analysis of variance (ANOVA) proved that both mean lutein and mean zeaxanthin concentrations of eggs from class 0 were statistically discriminable from mean lutein and zeaxanthin concentrations from eggs of all other classes ($P < 0.01$). Mean concentrations of synthetic xanthophylls in eggs of classes 1 (free range), 2 (barn), and 3 (cage) were as follows: canthaxanthin, 707 ± 284 μg/100 g; β-apo-8′-carotenoic acid ethyl ester, 639 ± 391 μg/100 g; and citranaxanthin, 560 ± 231 μg/100 g. Experiments with boiled eggs proved that β-apo-8′-carotenoic acid ethyl ester was the xanthophyll with the highest stability, whereas lutein was degraded to the largest extent (loss of 19%). Detailed knowledge about the xanthophyll amounts in eggs is indispensable to calculate the human uptake.

KEYWORDS: Egg yolk; lutein; zeaxanthin; HPLC; xanthophylls; LC-(APCI)MS

INTRODUCTION

The xanthophylls lutein and zeaxanthin have attracted a lot of interest since it was presumed that an increased nutritional uptake of both compounds may prevent adult macula degeneration (AMD) and age-related cataract formation (*1*−*3*). AMD is actually the main cause of loss of vision in Western countries. Besides dark-green leafy vegetables, egg yolks serve as a traditional source of xanthophylls (*4*) and are regarded as a highly bioavailable source of especially lutein and zeaxanthin (*5*). As only fat-soluble compounds are suitable to stain the egg yolk efficiently and consumers associate an intense color of the egg yolk with high quality, xanthophylls have been used to fortify poultry feed (*6*). The economic importance of the egg yolk to serve as a carrier for nutrients with an added value ("functional food") is demonstrated by ongoing efforts to create "designer eggs" comprising lutein, vitamin E, selenium, and others (*7*).

Currently, eight xanthophylls are permitted to be added to the feed of poultry (P) or laying hens (LH) in the European Union (EU). Those xanthophylls possess different carbon chain lengths (C_{30}−C_{40}) and functional groups (**Figure 1**): lutein (C_{40}; P), capsanthin (C_{40}; P), zeaxanthin (C_{40}; P), β-apo-8′-carotenal (C_{30}, aldehyde; P), canthaxanthin (C_{40}, diketone; P), β-apo-8′-carotenoic acid ethyl ester (C_{30}, ester; P), β-cryptoxanthin (C_{40}; poultry; P), and citranaxanthin (C_{33}, ketone; LH) (8). As the term "poultry" covers both fields of application, eight xanthophylls may be present in commercial egg yolks. The maximum concentration allowed in the feed was set at 80 mg/kg for all xanthophylls with only one exception: if canthaxanthin is added to the feed of laying hens, a maximum amount of 8 mg/kg is allowed. This regimentation is due to an unwanted side effect of canthaxanthin application. In extremely high dosages, minute crystals may be formed in the retina (*9*, *10*). Lutein, capsanthin, zeaxanthin, and β-cryptoxanthin are natural xanthophylls, occurring in various plants of commercial interest; the other xanthophylls are actually produced as bulk products by chemical synthesis. β-Carotene, the most abundant carotene in plants, is found only as a minor component in egg yolks because hens possess enzymes to convert β-carotene efficiently to vitamin A (*11*). Commercial eggs are classified in the EU according to the rearing method (classes: 0, ecological; 1, free range; 2, barn; 3, cage), the grade (A extra, A, B), and the size (S, below 53 g; M, 53 to <63 g; L, 63 to <73 g; XL, ≥73 g) (*12*).

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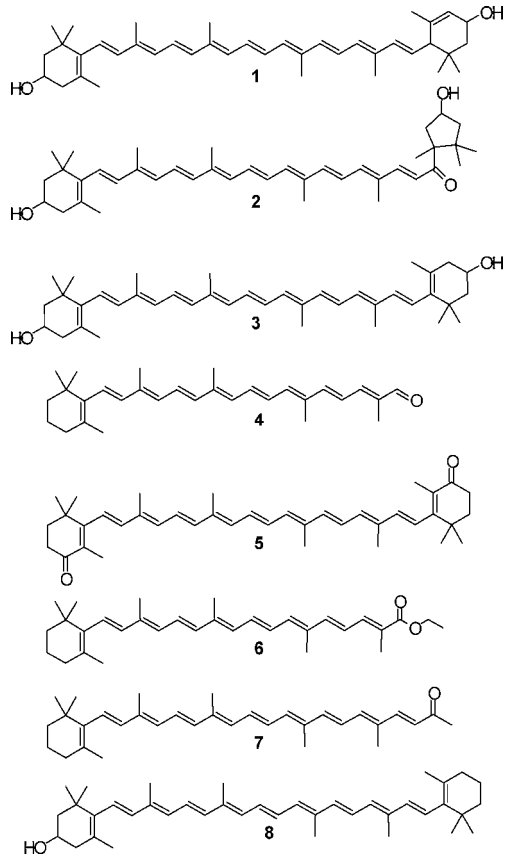

Figure 1. Chemical structures of xanthophylls used in poultry feeding: (**1**) lutein; (**2**) capsanthin; (**3**) zeaxanthin; (**4**) β-apo-8′-carotenal; (**5**) canthaxanthin; (**6**) β-apo-8′-carotenoic acid ethyl ester; (**7**) citranaxanthin; (**8**) β-cryptoxanthin.

Despite its widespread use, there is a lack of knowledge concerning the xanthophyll pattern of commercial egg yolks. Furthermore, little is known about the stability of xanthophylls with nutritional benefit during household cooking. Most data published refer to the concentrations achieved in yolks produced during pigmentation trails (see, e.g., refs *13* and *14*). Actually, none of the methods according to §64 LFGB allow for the individual analysis of xanthophylls in eggs (*15*). Likewise, the *Official Methods of Analysis of the AOAC* lists two methods to determine the sum of xanthophylls by spectroscopy (*16a*). In the scientific literature, further methods based on chromatography on open alumina columns (*14*) or high-performance liquid chromatography (HPLC) analysis (see, e.g., refs *18*−*22*) have been proposed. With respect to LC-MS analyses, only two studies describe the identification of xanthophylls in egg yolks: Liu et al. used HPLC-MS/MS to identify rhodoxanthin isomers in red egg yolks of Chinese ducks (*23*), and Kang et al. determined lycopene by liquid chromatography−mass spectrometry using an atmospheric pressure chemical ionization interface [LC-(APCI)MS] in a feeding trial using lycopene-fortified diets (*24*). To the best of our knowledge, no LC-(APCI)MS method allowing for unambiguous identification of all xanthophylls used in current poultry feeding has been published.

Taken together, the main purpose of the present study was to obtain detailed information about the xanthophyll concentrations present in commercial egg yolks before and after household cooking. Therefore, a sophisticated HPLC-diode array detection (DAD) method was developed, and peak identification was ascertained by LC-(APCI)MS analysis.

MATERIALS AND METHODS

Chemicals. Light petroleum ether (boiling fraction 40−60 °C), methanol, acetone, ethyl acetate, ethanol, and silica gel 60 (0.063−0.200 mm) were purchased from Merck (Darmstadt, Germany); *tert*-butyl methyl ether (TBME), sodium sulfate (anhydrous, >99%), and toluene were from Sigma-Aldrich (Taufkirchen, Germany). High-purity water was prepared with a Milli-Q 185 Plus water purification system (Millipore, Eschborn, Germany). All solvents were distilled before use.

Reference Compounds. Zeaxanthin was isolated by open column chromatography from a saponified wolfberry (*Lycium barbarum*) extract as described earlier (*25*) (wolfberries were kindly provided by Rich Nature Nutraceutical Labs, WA). Lutein was obtained from saponified marigold (*Tagetes erecta*) oleoresin in accordance with the same protocol (*25*) (marigold oleoresin was kindly provided by Euram Food, Stuttgart, Germany). β-Apo-8′-carotenal (>96%), canthaxanthin (>98%), and β-apo-8′-carotenoic acid ethyl ester (>80%) were obtained from Fluka (Taufkirchen, Germany); β-cryptoxanthin and capsanthin were generously provided by DSM (Kaiseraugst, Human Nutrition, Switzerland); and citranaxanthin was purchased from CaroteNature (Lupsingen, Switzerland).

Preparation of Samples. *Samples.* Seven batches of eggs from each type of husbandry (classes 0−3; size L, quality A, each) were obtained from local supermarkets. Analyses were usually performed not later than 2 weeks before the date of expiry.

Extraction of Fresh Egg Yolks. Eggs were cracked and the yolks separated from the egg white manually. Three egg yolks of each batch were pooled and homogenized using a spatula. Aliquots (4.5 g) were extracted four times using a ternary solvent mixture (light petroleum/ethyl acetate/methanol, 1:1:1, v/v/v; 15 mL each). To assist phase separation, distilled water (2 mL) was added. The supernatants were collected in a round-bottom flask, ethanol (2 mL) was added to remove traces of water, and the solvent mixture was evaporated to dryness (50 mbar, 30 °C, 10 min). Because of high amounts of lipids remaining after evaporation, the oily residue was completely transferred to a volumetric flask (10 mL) and made up to the volume with TBME/methanol (1:1, v/v). An aliquot was membrane filtered (0.45 μm) and immediately subjected to HPLC-DAD analysis. All work was performed under dim light. Concentrations were calculated as follows: c (μg/100 g of egg yolk) = concentration in the final solution (mg/L) × 1000/4.5. All analyses were performed in triplicate.

Extraction of Boiled Egg Yolks. Five eggs out of several batches were cooked for 10 min in sparkling boiling water (98.5 °C). The eggs were cooled with water (16.5 °C) and stored overnight in a refrigerator at 4 °C. Egg yolks were separated from the egg white, weighed, and homogenized with a blender (Moulinex Illico, Radolfzell, Germany; 10 s). Extraction of an aliquot (4.5 g) and HPLC-DAD analyses were performed as described above. In additional experiments, solid sodium sulfate (4.5 g) was added to homogenized egg yolk samples to increase the sample surface before extraction. To estimate the loss of weight of the egg yolk during cooking, additional egg yolks of the same batch were weighed without cooking, too.

Cleanup of Extracts for LC-(APCI)MS Analyses. Because of high amounts of lipids, egg yolk samples had to be fractionated on silica gel prior to LC-(APCI)MS analyses. To obtain an extract high in xanthophyll concentration, three extracts originating from the same batch were combined. The solvent was evaporated (50 mbar, 30 °C, 10 min), and the residue was dissolved in light petroleum ether (20 mL) and subjected to semipreparative open column chromatography (glass column, 400 × 20 mm) using silica gel (10 g) suspended in light petroleum ether as stationary phase. To remove lipids, the column was flushed with light petroleum ether (200 mL). For elution, mixtures of acetone in light petroleum ether [v/v; 1:99 (80 mL), 5:95 (80 mL), 10:90 (80 mL), 20:80 (100 mL), 30:70 (100 mL)] were used. Colored fractions were combined, the solvent was evaporated (50 mbar, 30 °C, 10 min), and the residue was dissolved in TBME/methanol (1:1, v/v; 10 mL). After membrane filtration (0.45 μm) an aliquot was subjected to LC-(APCI)MS analysis.

Xanthophyll Reference Solution for Method Development. Small amounts (2−5 mg) of commercial standards were dissolved in volumetric flasks in TBME/methanol (1:1, v/v; 100 mL). Aliquots of

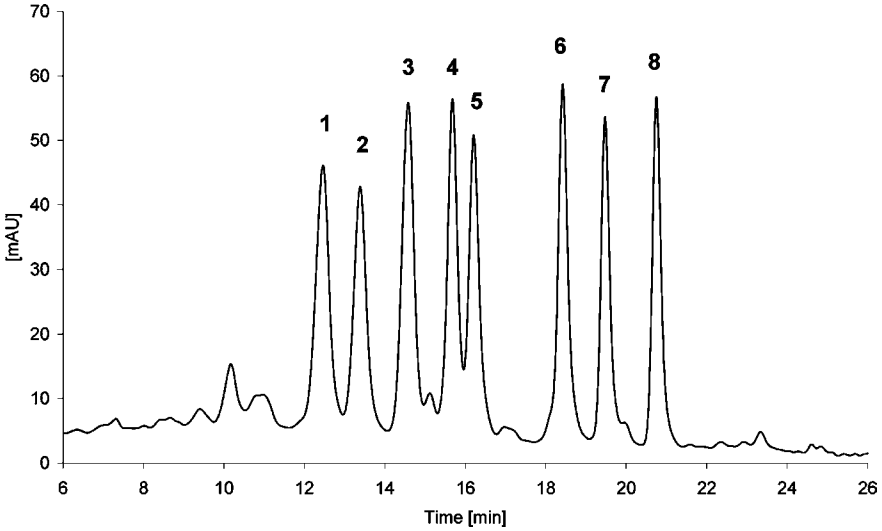

Figure 2. Representative HPLC chromatogram (extended section; 450 nm DAD) of a carotenoid reference solution comprising all xanthophylls used in poultry feeding. Peak numbers correspond to the assignments given in **Figure 1**.

the resulting stock solutions as well as lutein and zeaxanthin reference solutions were analyzed by HPLC-DAD. To obtain a reference solution comprising all relevant xanthophylls, aliquots of the stock solutions corresponding to a peak height of 50−60 mAU (relative to a final volume of 10 mL) were mixed in a round-bottom flask. The solvent was evaporated (50 mbar, 30 °C, 10 min) and the residue dissolved in TBME/methanol (1:1, v/v; 10 mL). The final solution was subjected to HPLC-DAD and LC-(APCI)MS analysis.

Quantification of Xanthophylls. *Calibration*. Calibration was performed in the range of 0.05−35.70 mg/L using dilutions of the respective stock solutions. Calibration graphs were recorded by plotting the respective peak areas [450 nm, (mAUs)] vs the concentrations (mg/L). Due to similar molar extinction coefficients, the same graph was applied for the quantification of both lutein and zeaxanthin. Limits of quantitation (LOQ) and determination (LOD) (micrograms per 100 g of egg yolk) were calculated from the calibration graphs according to the recommendations of the Deutsche Forschungsgemeinschaft (*26*) and were based on a sample amount of 4.5 g, a final volume of 10 mL, and an injection volume of 20 μL (**Table 3**).

Validation of the Extraction Method. The reproducibility of the method was investigated by spiking homogenized fresh egg yolk samples free of synthetic xanthophylls (class 0; sample 0f, **Table 2**) with aliquots of a β-apo-8′-carotenal stock solution (c = 19.5 μg/mL in ethanol; 1 mL each), resulting in a concentration typically found in egg yolks (433 μg/100 g). The spiked samples were extracted and analyzed by HPLC-DAD. Recoveries were calculated on the basis of AOAC methods (*16b*) as follows: % recovery = (measured concentration in fortified material − measured concentration in unfortified material; set to zero) × 100/known increment in concentration. The following recovery was calculated: 99.1 ± 0.6% (n = 3).

HPLC and LC-(APCI)MS: Apparatus and Conditions. A modular HP1100 (Hewlett-Packard GmbH, Waldbronn, Germany) system with a diode array detector (450 nm) was used for analysis of the xanthophylls. For separation, a YMC analytical column (YMC Europe GmbH, Dinslaken, Germany) with 5 μm C30-reversed phase material (250 × 4.6 mm i.d.) including a precolumn (10 × 4.0 mm i.d.) was used and kept at 35 °C. The mobile phase consisted of methanol (A) and a mixture of methanol/*tert*-butyl methyl ether/water [6:90:4 v/v/v (B)], using gradient elution (%A/min, 99/0, 90/10, 70/20, 0/30, 99/35, 99/40; 1 mL/min, 20 μL). LC-(APCI)MS was performed on an HP1100 modular HPLC system, coupled to a Micromass (Manchester, U.K.) VG platform II quadrupole mass spectrometer using an APCI interface (operated in the positive mode). The APcI source was heated at 150 °C, and the APcI probe was kept at 400 °C. The corona voltage was set to 3.2 kV, the HV lens to 0.5 kV, the skimmer offset to 5 V, and the cone to 30 V. Nitrogen was used as both sheath and drying gas at 75 and 300 L/h, respectively. Mass spectra were acquired within a scan range of m/z 300−700 (scan time, 2.0 s; interscan delay, 0.1 s). UV−vis spectra were recorded from 240 to 600 nm (interval 2.0 nm). Data were processed with MassLynx 3.2 software. The eluents, the flow, and the injection volume were identical to those used for HPLC-DAD analyses. Further MS calibration parameters have been detailed earlier (*27*).

Statistical Analysis. Quantitative data were presented as means ± SD of at least triplicate experiments. Analysis of variance (ANOVA) was performed on the data obtained using Microsoft Excel XP software. The significant statistical level was set to $P < 0.01$ (two-sided F and t tests).

RESULTS AND DISCUSSION

Optimization of the HPLC Method. For development of a reliable HPLC method, a reference solution comprising all xanthophylls used in poultry feeding was generated. As the molar extinction coefficients of individual xanthophylls are different (*28*), the resulting peak areas of a solution comprising all compounds with the same concentration will be remarkably different. Thus, one reference solution containing all compounds with the same peak height (50−60 mAU at 450 nm), irrespective of the individual concentration, was established. This solution allowed for optimal evaluation of the peak separation efficiency of the HPLC method. Chromatographic separation was performed using an RP-C30 phase as well as pure methanol and a mixture of methanol, TBME, and water for gradient elution. A representative HPLC chromatogram (total running time = 40 min) is depicted in **Figure 2**, proving the suitability of the method to separate all compounds within 10 min simultaneously. With the exception of β-apo-8′-carotenal (**4**) and canthaxanthin (**5**), baseline separation was achieved for all xanthophylls. As β-apo-8′-carotenal was not present in commercial egg yolk samples, the peak overlapping of **4** and **5** was regarded as tolerable. Remarkably, peak separation of lutein (**1**) and zeaxanthin (**3**) was exceptionally good (retention time difference of 2.12 min, peak width at the basis = 1.00 min each; resolution R = 2.12), indicating a high separation efficiency that may be used in xanthophyll research. Furthermore, capsanthin (**2**) is well separated from lutein (**1**), two xanthophylls that usually coelute in our HPLC system using C30 phases. Small peaks appearing in the standard chromatogram were attributed to (Z)-isomers, already present in the reference compounds. Because the UV−vis absorption spectra of individual xanthophylls were found to be spectroscopically pure, we concluded that (Z)-isomers possibly coeluting with (E)-isomers were present in rather low

Table 1. Spectroscopic and LC-(APCI)MS Data (Exact Masses) Used for Identification of Xanthophylls Used in Poultry Feeding (Numbering of Xanthophylls Corresponds to **Figure 1**)

xanthophyll	VIS maxima[a] (nm); ϵ [L/(mol × cm)] [solvent – (nm)][b]	main ions (*m*/*z*; intensity)
lutein (**1**)	420/446/472; 145100 (ethanol – 445)	569.4 (14%) $[M + H]^+$, 551.4 (100%) $[M + H - H_2O]^+$
capsanthin (**2**)	474; 121000 (toluene – 483)	585.4 (100%)
zeaxanthin (**3**)	426/452/478; 144500 (ethanol – 450)	569.4 (100%), 551.4 (15%) $[M + H - H_2O]^+$
β-apo-8′-carotenal (**4**)	460; 110000 (light petroleum ether – 457)	417.3 (100%)
canthaxanthin (**5**)	476; 107300 (light petroleum ether – 463)	565.4 (100%)
β-apo-8′-carotenoic acid ethyl ester (**6**)	446; 87700 (light petroleum ether – 430)	461.3 (100%)
citranaxanthin (**7**)	474; 98000 (light petroleum ether – 463)	457.3 (100%)
β-cryptoxanthin (**8**)	426/452/478; 131000 (light petroleum ether – 452)	553.4 (100%)

[a] Determined in the HPLC eluents. [b] Individual values according to ref *28*.

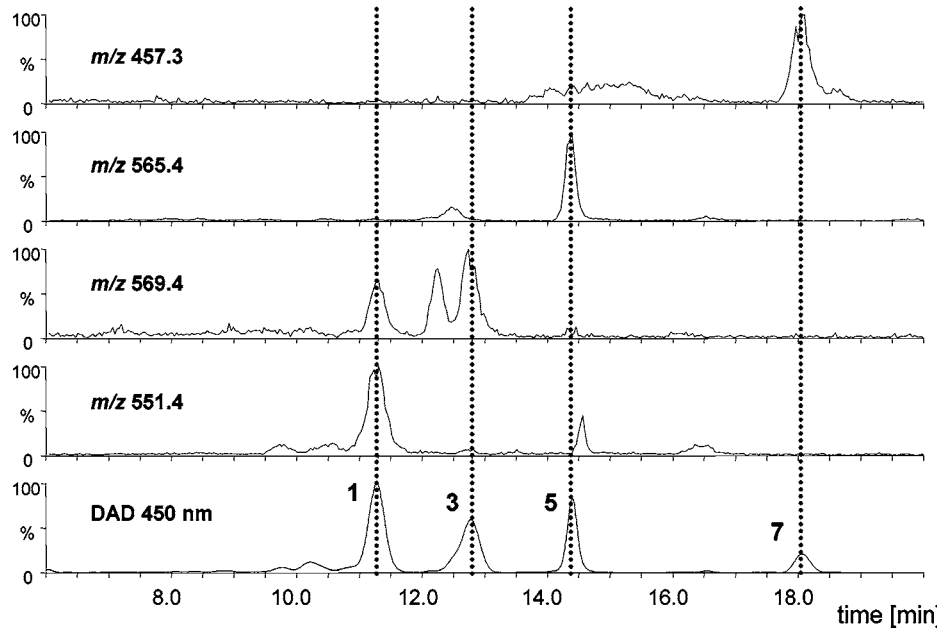

Figure 3. LC-(APCI)MS analysis (extended section) of an egg yolk extract (sample 2c, **Table 2**), cleaned by open column chromatography. The bottom trace corresponds to detection at 450 nm (DAD). The mass traces allow for identification of lutein (**1**, *m*/*z* 551.4), zeaxanthin (**3**, *m*/*z* 569.4), canthaxanthin (**5**, *m*/*z* 565.4), and citranaxanthin (**7**, *m*/*z* 457.3). Peak numbers correspond to the assignments given in **Figure 1**.

amounts. Taken together, the separation efficiency was high enough to allow for unambiguous xanthophyll identification in routine analyses. Detection was performed exclusively at 450 nm (DAD) for all xanthophylls, although the absorption maxima of red xanthophylls show a bathochromic shift of up to 26 nm (e.g., canthaxanthin, 476 nm; **Table 1**). Typical absorption spectra of apocarotenals or xanthophylls with keto groups show a broad plateau of absorbance rather than distinct maxima (superposition of the vibration fine structure), leading to a negligible loss of sensitivity when detection is performed in the slope (e.g., canthaxanthin, −14%). Furthermore, the use of only one detection wavelength simplifies data processing in daily routine analysis.

Identification of Xanthophylls in the Reference Solution by LC-(APCI)MS. For unequivocal xanthophyll identification, LC-(APCI)MS was used. Because the interface was operated in the positive mode, only positively charged ions were detected. The data set for identification of the respective xanthophylls (full scan) present in the reference solution is given in **Table 1**. Besides lutein (**1**), all xanthophylls formed intense quasimolecular ions ($[M + H]^+$) by protonation of the oxygen atoms of hydroxyl or keto groups. To scan for lutein (**1**), *m*/*z* 551.4 was used since this mass represents the backbone of lutein easily formed by the loss of water ($[M + H - H_2O]^+$) from the respective quasimolecular ion (*m*/*z* 569.4) (*27*). The expected loss of ethanol from the quasimolecular ion of β-apo-8′-carotenoic acid ethyl ester (**6**) did not occur under the LC-MS conditions applied. Consequently, the quasimolecular ion (*m*/*z* 461.3) was used for identification. Canthaxanthin (**5**) was the only component forming an intense sodium adduct ion ($[M + Na]^+$, 40%; *m*/*z* 587.4), which was not used for identification. In general, UV−vis maxima obtained by DAD were used to facilitate peak assignment.

Identification of Xanthophylls in Egg Yolk Samples by LC-(APCI)MS: LC-(APCI)MS Analysis of Egg Yolk Extracts. To ensure an unambiguous peak identification, representative sample extracts were analyzed by LC-(APCI)MS. Due to the high lipid content of the egg yolk extracts, a cleanup step using open column chromatography on silica gel was performed. **Figure 3** shows a representative example of an LC-(APCI)MS analysis of one egg yolk extract (class 2) comprising lutein, zeaxanthin, canthaxanthin, and citranaxanthin. The mass traces useful for xanthophyll identification were extracted from the total ion chromatogram (TIC) after time alignment. Lutein was identified on the basis of the intense signal at 551.4 Da and a signal with lower intensity at 569.4 Da; in the case of zeaxanthin (**3**) peak ratios were inverse. In front of the zeaxanthin peak an additional compound was found. Due to the peak broadening caused by the transfer line between the HPLC and mass spectrometer, neither compound was baseline separated. In contrast, the minor peak was clearly separated in HPLC-DAD analyses. Consequently, this compound did not

Table 2. Concentrations of Xanthophylls Determined in the Yolk of Eggs Obtained from Local German Supermarkets (Mean Values ± Standard Deviations; $n = 3$)[a]

	μg/100 g of egg yolk					
sample	lutein	zeaxanthin	canthaxantin	β-apo-8′-ethyl ester	citranaxanthin	β-cryptoxanthin
0a	2057.0 ± 4.3	1287.5 ± 17.9				90.6 ± 2.0
0b	1541.6 ± 17.8	1015.7 ± 14.3				85.6 ± 2.1
0c	1460.5 ± 22.2	1235.0 ± 9.5				104.3 ± 7.1
0d	2477.7 ± 28.7	984.0 ± 4.6				71.0 ± 0.3
0e	1273.6 ± 2.3	781.5 ± 0.7				61.4 ± 6.6
0f	1512.0 ± 10.8	774.9 ± 8.7				73.3 ± 3.4
0g	2026.1 ± 3.0	1071.5 ± 12.9				91.0 ± 5.4
1a	575.8 ± 1.3	221.2 ± 10.9	448.2 ± 10.3	474.5 ± 7.2		
1b	572.8 ± 13.4	220.7 ± 7.7	321.3 ± 11.3	140.2 ± 4.6		
1c	776.7 ± 13.4	382.4 ± 3.1		trace	710.6 ± 4.6	trace
1d	1130.8 ± 34.2	434.0 ± 2.5	990.3 ± 11.9			trace
1e	554.7 ± 25.8	360.2 ± 15.2	910.7 ± 16.3			trace
1f	534.0 ± 15.3	377.9 ± 21.3	546.9 ± 9.2		294.6 ± 0.1	trace
1g	1097.8 ± 23.6	281.6 ± 10.0	502.3 ±13.5			trace
2a	310.0 ± 20.2	160.0 ± 8.5	468.4 ± 0.2	478.1 ± 2.6		
2b	924.1 ± 1.4	209.6 ± 4.3	948.7 ± 11.0			
2c	537.0 ± 31.1	356.0 ± 17.2	1107.1 ± 25.8		676.0 ± 12.7	trace
2d	832.1 ± 20.5	249.2 ± 8.8	1123.1 ± 4.6			trace
2e	132.4 ± 8.5		539.8 ± 10.1	534.8 ± 17.7		
2f	567.9 ± 11.0	121.8 ± 7.4	1119.0 ± 7.0			
2g	483.6 ± 15.1	235.5 ±16.2	809.8 ± 6.5	1415.6 ± 5.4		
3a	1180.7 ± 6.6	496.3 ± 10.3	1156.9 ± 4.4			68.8 ± 1.7
3b	225.8 ± 9.3		369.4 ± 0.5	199.2 ± 3.9		
3c	220.6 ± 3.0	trace	555.6 ± 3.9	865.9 ± 2.3		
3d	116.8 ± 3.3		599.8 ± 0.1	605.8 ± 6.0		
3e	599.2 ± 4.0	300.1 ± 0.5	646.8 ± 10.1	1100.4 ± 15.6		
3f	356.3 ± 12.9		377.9 ± 6.2			
3g	174.6 ± 0.4		603.6 ± 16.0	575.5 ± 6.4		

[a] Samples were grouped according to husbandry classes: 0, ecological; 1, free range; 2, barn; 3, cage; a–g indicate different batches; trace, value between LOD and LOQ.

Table 3. Calibration Graphs and Limits of Quantitation (LOQ) and Determination (LOD) (Based on a Final Volume of 10 mL, a Sample Amount of 4.5 g, and an Injection Volume of 20 μL) Used for Quantification of Xanthophylls in Egg Yolks by HPLC-DAD

	calibration range (mg/L)	calibration graphs *c* (mg/L)	LOQ (μg/100 g)	LOD (μg/100 g)
lutein[a]	0.05–10.15	[area (mAUxs) − 38.10]/243.59	84.9	56.7
canthaxanthin	1.16–23.16	[area (mAUs) + 4.15]/163.31	114.1	76.1
β-apo-8′-carotenoic acid ethyl ester	0.60–35.70	[area (mAUxs) + 61.28]/210.12	127.5	53.2
citranaxanthin	0.27–5.42	[area (mAUxs) + 6.09]/132.01	46.7	25.2
β-cryptoxanthin	0.54–10.75	[area (mAUxs) + 17.44]/225.12	52.7	26.4

[a] The same calibration graph was used for the calculation of zeaxanthin.

hamper zeaxanthin quantification. The absorption spectrum showed a broad maximum at 465 nm and an additional peak at 370 nm (*cis*-band), and LC-(APCI)MS analyses revealed a mass of 565.4 Da. The peak was not found in eggs of class 0 or in sample 1c (samples free of canthaxanthin; **Table 2**). Thus, it was concluded that this peak may represent a (Z)-isomer of canthaxanthin. *all*-(*E*)-canthaxanthin (**5**) and -citranaxanthin (**7**) were identified on the basis of their respective quasimolecular ions (*m*/*z* 565.4 and 457.3) and their characteristic UV−vis spectra. In the slope of the *all*-(*E*)-canthaxanthin peak, *m*/*z* 551.4 was present additionally, pointing to a lutein-derived compound, for example, anhydrolutein. This peak overlapping may result in a marginal overestimation of the canthaxanthin concentration if peak integration at 450 nm is not done properly. Experiments to separate this compound by changing the eluents or modifying the column temperature failed. In the mass trace corresponding to citranaxanthin (*m*/*z* 457.3) a peak with the same mass and an enhanced retention time may be due to a (Z)-isomer.

Quantification of Xanthophylls in Egg Yolk Samples. *Native Egg Yolks*. The extraction of lipids and lipid-soluble compounds from egg yolk is usually accomplished by nonpolar solvents. In this study a solvent mixture comprising light petroleum ether, ethyl acetate, and methanol, which already was successfully applied in earlier studies for the extraction of xanthophylls from plants (*29*), was used. Addition of this mixture to egg yolk samples resulted in rapid protein denaturation and allowed for quick formation of a colored upper layer comprising the xanthophylls. Because the extract contained additionally high amounts of lipids and lipid-soluble components, the residue remaining after evaporation had to be transferred completely to a volumetric flask (10 mL) to avoid errors due to the volume of coextracted lipids. Representative egg yolk samples were obtained by mixing three egg yolks out of each batch. Calibration graphs, LOQs, and LODs of compounds determined in this study are given in **Table 3**. Concentrations of xanthophylls found between LOQ and LOD were designated "traces".

With respect to individual husbandry classes, the following quantitative results were obtained (**Table 2**): Lutein was the predominant xanthophyll present in commercial egg yolks and

Table 4. Statistical Data [Mean Value ± Standard Deviation (Micrograms per 100 g of Egg Yolk)], Minimum–Maximum Concentrations (Micrograms per 100 g of Egg Yolk), and Number of Batches of Xanthophylls Present in Commercial Egg Yolks of Husbandry Classes 0–3

	husbandry class			
	0	1	2	3
lutein	1764.1 ± 430.0	748.9 ± 262.4	541.0 ± 275.6	410.6 ± 375.0
	1273.6–2477.7	534.0–1130.8	132.4–924.1	116.8–1180.7
	7	7	7	7
zeaxanthin	1021.4 ± 199.5	325.4 ± 84.4	222.0 ± 81.1	398.2 ± 138.7
	774.9–1287.5	220.7–434.0	121.8–356.0	300.1–496.3
	7	7	6	2
canthaxanthin		620.0 ± 268.2	873.7 ± 277.6	615.7 ± 263.0
		321.3–990.3	468.4–1123.1	369.4–1156.9
		6	7	7
β-apo-8′-carotenoic acid ethyl ester		707.4 ± 236.4	809.5 ± 525.7	669.4 ± 338.5
		140.2–474.5	478.1–1415.6	199.2–1100.4
		2	3	5
citranaxanthin		502.6 ± 294.2	676.0 ± 0	
		294.6–710.6		
		2	1	
β-cryptoxanthin	82.5 ± 14.6			68.8 ± 0
	61.4–104.3			
	7			1

was found in each sample. In accordance with legal regimentations, lutein and zeaxanthin—together with small amounts of β-cryptoxanthin—were the only xanthophylls present in eggs of class 0 (ecological husbandry). Lutein and zeaxanthin are natural xanthophylls typically present in dark-green leafy plants, serving as diets for laying hens housed under ecological conditions. The presence of β-cryptoxanthin can be a consequence of feeding corn. This assumption is assisted by the high concentration found in sample 3a: according to the label on the package, these chickens were fed with corn.

Statistical data (mean value ± standard deviation; minimum−maximum concentration; number of samples) of xanthophylls present in commercial egg yolks are presented in **Table 4**. ANOVA proved that both mean lutein and mean zeaxanthin concentrations of eggs from class 0 were statistically discriminable from mean lutein and zeaxanthin concentrations from eggs of all other classes (two-sided F and t test, $P < 0.01$). Mean lutein and zeaxanthin concentrations of eggs from classes 1−3 were not discriminable among themselves on the same level of significance. Comparison of the mean lutein concentration with those found in other classes ascertained a decrease in the order of class 0 to class 3 (**Table 4**). In egg yolks of the rearing classes 1−3, additional synthetic xanthophylls were present: canthaxanthin was found in 95% of these samples, and β-apo-8′-carotenoic acid ethyl ester and citranaxanthin were present in 48 and 14%, respectively (mean values are given in **Table 4**). Further statistical analysis was not applied to the data set as addition of synthetic xanthophylls to poultry feed does not differ among husbandry classes 1−3 (*8*, *12*), and no attempts were made to differentiate eggs from classes 1−3 on the basis of synthetic xanthophyll concentrations.

As only limited information is available, comparison of the xanthophyll concentrations with data published is difficult. Majchrzak and Elmadfa analyzed commercial eggs produced under different rearing conditions (*20*). They found highest lutein and zeaxanthin concentrations in winter eggs from a free range system; values were given as micrograms per 100 g of whole egg. Taking into account that the yolk comprises roughly 27% of the egg content (*30*), lutein concentrations were in the range of 1400−2900 μg/100 g of egg yolk. Using the same calculation, Bonomi et al. (*22*) determined lutein concentrations ($n = 2$) of 1400−1500 μg/100 g of egg yolk, and Handelman et al. found 1500 μg of lutein/100 g of egg yolk in representative samples used in a human intervention study (*5*). Ollilainen et al. calculated the lutein concentration in egg yolks as part of a composition study of Finnish food and gave a lutein concentration of 1576 μg/100 g of egg yolk (*18*). Those results are in accordance with lutein concentrations of eggs of class 0 (1274−2478 μg/100 g). The presence of the synthetic xanthophylls in eggs of classes 1−3 as well as their absence in "ecological eggs" is in accordance with legal regimentations. Although capsanthin and β-apo-8′-carotenal are allowed as feed additives and capsanthin may be applied as "red pepper flour" in ecological rearing, neither xanthophyll was found in any egg yolk investigated.

Boiled Egg Yolks. Comparison of the weight of egg yolks before and after boiling (samples 1g, 2e, 2f, 2g, **Table 2**; $n = 3$ each) revealed a loss of weight during cooking of 3.5 ± 1.3%. As this loss was regarded as negligible, xanthophyll concentrations in egg yolks calculated before and after boiling (samples 0c, 0d, 2f, 2g, 3c, 3d, 3e, **Table 2**; $n = 3$ each) were compared without correction factor. Remarkably, no additional peaks [e.g., (Z)-isomers] appeared in the HPLC chromatograms of extracts of boiled eggs. However, the following losses of xanthophylls were calculated: lutein, 19 ± 15%; zeaxanthin, 15 ± 11%; canthaxanthin, 12 ± 7%; and β-apo-8′-carotenoic acid ethyl ester, 10 ± 1%. These results are in contrast to those of Surai et al. (*7*), who stated that the concentration of lutein in "designer eggs" does not decrease during boiling. The addition of solid sodium sulfate to boiled and homogenized egg yolk samples had no influence on the extractability: losses were found to be in the same range (13−24%). Again, β-apo-8′-carotenoic acid ethyl ester was found to be the xanthophyll with the highest stability, whereas lutein was degraded to the largest extent. Thus, the degradation of xanthophylls during household cooking of eggs can be estimated to be roughly between 10 and 20%.

The results obtained in this study are indispensable to the calculation of the egg-based xanthophyll intake in the average human diet. Furthermore, knowledge about xanthophyll concentrations in eggs is useful to estimate whether the concentration found in a specific food is due to the use of egg yolk as a single xanthophyll source or if a—possibly illegal—addition of food dyes has to be verified.

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