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# A Simplified and Small-Scale Sample Preparation Technique for Determining Astaxanthin, Canthaxanthin, and β-Apo-8′-Carotenoic Acid Ethyl Ester in Hen's Egg Yolk

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**Summary.** This paper described a fast, simple, and small scale method of sample preparation followed by high-performance liquid chromatography (HPLC) coupled photodiode array (PDA) detector for simultaneous quantification of astaxanthin, canthaxanthin, and β-apo-8′-carotenoic acid ethyl ester in hen's egg yolk. The HPLC-PDA was performed on a C18 column with an isocratic mobile phase. Analytes were extracted from the sample using a handheld ultrasonic homogenizer, and purified by MonoSpin®-SI, a centrifugal monolithic silica spin mini-columns, and quantified within 30 min. The proposed method obtained average recoveries for the three analytes in the range of 70.5–101.1% with relative standard deviations ≤5.0%. The quantitation limits were 0.3 μg $g^{-1}$ for AX, 0.5 μg/g for CX, and 1.0 μg $g^{-1}$ for ACAEE, respectively.

**Key Words:** astaxanthin, canthaxanthin, β-apo-8′-carotenoic acid ethyl ester, centrifugal monolithic silica spin mini-column

## Introduction

Astaxanthin (AX), canthaxanthin (CX), and β-apo-8′-carotenoic acid ethyl ester (ACAEE) *(Fig. 1)* are coloring agents, xanthophylls, and widely used as feed additives to pigment the eggs or meat of poultry, salmon, and trout. The permitted CX and ACAEE are red and yellow pigments, respectively, and commonly added to feeds for laying hens in order to achieve the desired egg yolk color. Although the European Union (EU) permits eight xanthophylls to be added to the feed of chickens, the use of chemically synthesized CX and ACAEE is nearly the norm in the EU and Japan [1–5]. They are highly potent red/yellow colorants available to poultry industry. In comparison to other red/yellow xanthophylls, these are in a high bioavailability form and have a higher deposition rate in chicken egg yolk [5–8].

Eggs are a very important and basic food because they are highly nutritious, cheap, and readily available. It becomes a raw material of every processed food. The color of egg yolks greatly affects the purchasing behavior of the consumer [9]. CX and ACAEE are used to brighten the yolks of chicken

eggs in response to consumer demands. Since consumers associate bright product coloration with health and quality, CX and ACAEE are particularly important in the poultry-farming industry as their pigments for egg yolks.

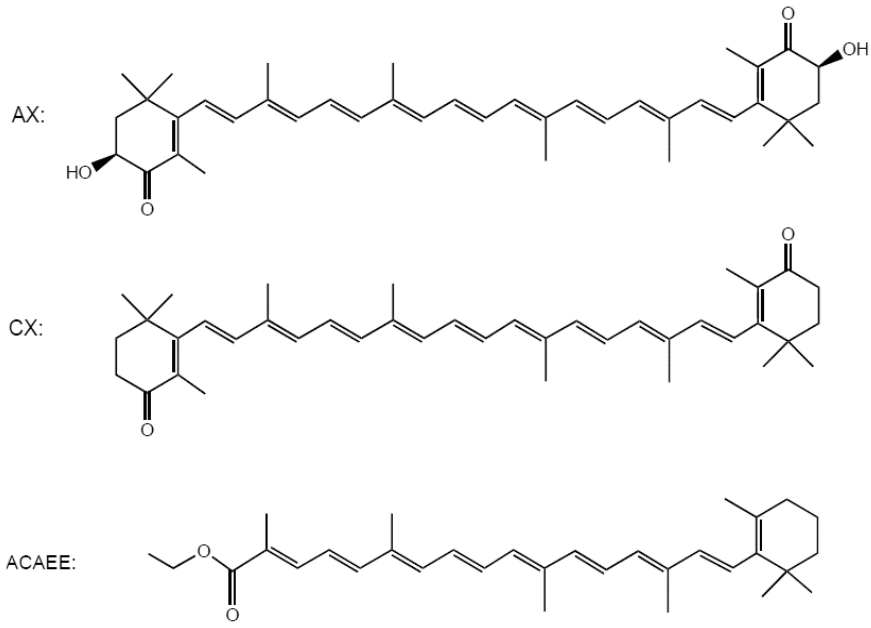


*Fig. 1.* Molecular structures of AX, CX, and ACAEE

Recent scientific findings indicate that AX is a powerful antioxidant and can serve as a potent free-radical scavenger. Moreover, AX has been found to provide many essential biological functions, including protection against lipid–membrane per oxidation of essential polyunsaturated fatty acids and proteins, DNA damage, and UV light effects; it also plays an important role in immunological defense [10]. With the view to the functions, AX-containing eggs are produced and sold now. Except these are eggs laid by the hens that were fed a diet supplemented with AX-rich krill or Phaffia rhodozyma [11–14] because AX is not permitted to be used in laying hens [1, 2, 4].

There is a requirement to monitor the human intake of the three pigments to corroborate that the acceptable daily intakes (ADIs) [1, 15, 16] are not exceeded. Besides the European Food Safety Authority (EFSA) [2] and the Japanese Ministry of Health, Labour and Welfare [17] have set maximum residue limits (MRLs) for the CX in several pigments in egg yolk at 30 and 25 μg $g^{-1}$, respectively, to ensure the safety and appropriateness of eggs

for human consumption. Monitoring the presence of the frequently used pigments in egg yolk is, therefore, a necessary means of guaranteeing food safety.

In answer to the present expansion in the internal food trade, the development of international standardized methods to determine chemical contents in foods is essential in order to guarantee equitable international trade in these foods and ensure food safety for consumers. Without regard for industrial nations and developing countries, the optimal harmonized method for chemical monitoring in foods must be simple, small scale, economical in time and cost, and must cause negligible harm to the environment and analysts. Several techniques based on high-performance liquid chromatography (HPLC) for the quantification of xanthophylls in egg yolk samples have been reported [18–21]. However, these methods cannot detect simultaneous AX, CX, and ACAEE; and they involve several analytical steps in the sample preparation, which are time- and cost-consuming and do not permit the determination of a large number of samples. Additionally, most of the recent methods are based on LC–MS/MS. The facility available is limited to part of industrial nations because these are hugely expensive, and the methodologies used are complex and specific. These are unavailable in a lot of laboratories for routine analysis, particularly in developing countries. No optimal method that satisfies the aforementioned requirements has yet been identified.

The present method was developed in such a way that it is idiot-proof and small-scale with minimized organic solvent consumption, and AX, CX, and ACAEE contents in an egg yolk can be determined with higher accuracy and precision.

# Experimental

## Reagents and Apparatus

Standards of astaxanthin (AX), canthaxanthin (CX), and β-apo-8′-carotenoic acid ethyl ester (ACAEE) were generous gifts from DSM Nutrition Japan, Co., Ltd. (Tokyo, Japan). Acetone and distilled water was of HPLC grade (Wako Pure Chem. Ltd., Osaka, Japan).

The following apparatuses were used in the sample preparation: handheld ultrasonic-homogenizer (model HOM-100, 2 mm ID probe, Iwaki Glass Co., Ltd., Funabashi, Japan); micro-centrifuge (Biofuge® fresco, Kendo Lab. Products, Hanau, Germany); a MonoSpin® as centrifugal monolithic silica spin mini-column (sample throughput volume: 300 μL), MonoSpin-SI (silica gel, bonded Si-OH, normal-phase mode) (GL Sciences, Inc., Tokyo, Japan). An Inertsil® ODS-4 (C18) (5 μm $d_p$, 150 × 4.6 mm) (Pore diameter, 10 nm;

Pore volume, 1.05 mL $g^{-1}$; Surface area, 450 $m^2$ $g^{-1}$; Carbon load, 11%) column for HPLC was used (GL Science).

The HPLC system, used for method development, included a model PU-980 pump and DG-980-50-degasser (Jasco Corp., Tokyo, Japan) equipped with a model CO-810 column oven (Thosoh Corp., Tokyo, Japan), as well as a model SPD-M10$A_{VP}$ photodiode-array (PDA) detector (Shimadzu Scientific Instruments, Kyoto, Japan).

## HPLC Operating Conditions

The analytical column was an Inertsil® ODS-4 (5 μm, 150 × 4.6 mm) column using an acetone–water (84:16, $v/v$) mobile phase at a flow rate of 1.0 mL $min^{-1}$ at 25°C. PDA detector was operated at 190–600 nm, the monitoring wavelength was adjusted to 470 nm which represents an average maximum for the analytes. The injection volume was 10 μL.

## Preparation of Stock Standards and Working Mixed Solutions

Stock standard solutions of AX, CX, and ACAEE were prepared by dissolving each of the analytes in acetone to a concentration of 100 μg $mL^{-1}$. Each solution was put into cryo-vial, sealed and stored at −20°C, and protected from light until further use. Working mixed standard solutions were freshly prepared by suitably diluting the stock solutions with acetone on the day of the analysis.

## Preparation of Calibration Standards and Quality Control Samples

For method validation studies, calibration standards and quality control samples (QCs), terms defined in the FDA guideline [22], were prepared by spiking appropriate aliquots of the mixed standard solution in blank egg yolk samples. Calibration standards were used to construct calibration curves from which the concentrations of analytes in unknown monitoring samples are determined practically. QCs used to evaluate the performance of the proposed method. In this study, the standards were prepared in the range of 1–50 μg $g^{-1}$ for all analytes. Three QC levels (for all analytes, QC1 = 5 μg $g^{-1}$; QC2 = 25 μg $g^{-1}$; QC3 = 30 μg $g^{-1}$) were prepared.

### Egg Yolk Samples

To obtain egg yolk samples for the present study, a total of 5 laying hens (White Leghorn, aged 30 weeks) that were kept in individual cages were used. Feed and water were given *ad libitum*. The pigment-free basal layer diet fed to the 5 hens continuously for 3 weeks. After the additive-free feeding, all of the eggs laid by hens were collected for 2 days. The albumen and egg yolks of each egg were separated immediately. The egg yolks were uniformed fully and used as blank egg yolk samples.

### Sample Preparation

An accurate 0.1-g sample was taken into a 1.5-mL micro-centrifuge tube and homogenized with 0.6 mL of 80% ($v/v$) acetone solution (in water) with a handheld ultrasonic-homogenizer for 30 s. After being homogenized, the capped tube was centrifuged at 10,000$g$ for 5 min. A 0.1 mL of supernatant liquid was poured to a MonoSpin®-SI and, immediately after the capped mini-column was centrifuged at 3000$g$ for 1 min. The eluate was injected into the HPLC system.

### Method Validation

The performance of the developed method was validated in terms of some parameters from the international guidelines for bio-analytical procedure [22–26].

## Results and Discussion

### Assay Conditions

As carotenoids are light-labile, the present assays were performed under a darkened room condition to protect against light exposure.

### Sample Preparation—Application of Centrifugal Spin Mini-Column

In comparison to the previous techniques for determining xanthophylls in egg yolk samples [18–20], the procedure used in this study is an easy and small-scale technique that minimizes organic solvent consumption in the preparation of AX, CX, and ACAEE. The extract obtained by the extracting operation was purified by subsequent centrifugal monolithic silica spin mini-column, MonoSpin®-SI. The spin mini-column is a monolithic SPE col-

umn which is said to be excellent for the small volume sample with easy and quick operation by centrifuge [27].

*Table I* presents the effect of acetone concentration in the eluent (acetone–water, *v*/*v*) on the recoveries of AX, CX, and ACAEE from MonoSpin®-SI. In this study, a 100-μL portion of a mixed standard solution containing 2.5 μg of each compound was applied to the spin mini-column. The eluate was examined by HPLC. Acetone solutions were >70% acetone in water as the eluent gave good recoveries for all analytes. There were no significant differences in data among 100–70% acetone eluents.

*Table I*. Effect of acetone concentration in the eluent on recoveries of AX , CX, and ACAEE from MonoSpin®-SI

| Compound | Recovery (%) | | | | |
|---|---|---|---|---|---|
| | Acetone (%, $v/v^{-1}$) in the eluent[a] | | | | |
| | 100 | 90 | 80 | 70 | 60 |
| AX | 96 | 101 | 99 | 98 | 59 |
| CX | 97 | 100 | 101 | 102 | 28 |
| ACAEE | 71 | 70 | 73 | 69 | 20 |

Data are averages ($n$ = 3). A 100-μL portion of a mixed standard solution containing 2.5 μg of AX and CX was applied to the MonoSpin®-SI.

[a]Acetone–water.

Based on the findings, egg yolk extracts processed with 0.6 mL of 100–70% acetone, respectively, were examined. The extract was fortified (25 μg $g^{-1}$ egg yolk sample of each compound) with a mixed standard solution, and mixed. A 100-μL portion of the extract was applied to MonoSpin®-SI. The centrifugal acceleration and time were standardized at 3000*g* and 1 min, respectively. The eluate was determined by HPLC, and the resulting chromatograms were compared with regard to the recoveries and purification efficacy. An 80% acetone as an extraction solution and the MonoSpin-SI eluent gave the best recovery of three analytes simultaneously and the most clear chromatogram without interfering peaks.

The present procedure can realize small-scale extraction and easy purification of AX, CX, and ACAEE in quite a short time and resulted in sufficient recoveries and repeatabilities *(Table II)*.

*Figure 2* displays the HPLC traces under the established procedure including the HPLC system. The resulting chromatograms were free of interfering compounds for quantitation and identification of AX, CX, and ACAEE by HPLC, with PDA detector set at 470 nm (giving an average maximum for the analytes). The present HPLC analysis accomplished good

separations without the need for a gradient system to improve the separation and pre-column washing after analysis. This figure demonstrates that the present method can provide the quantitation and identification of the analytes.

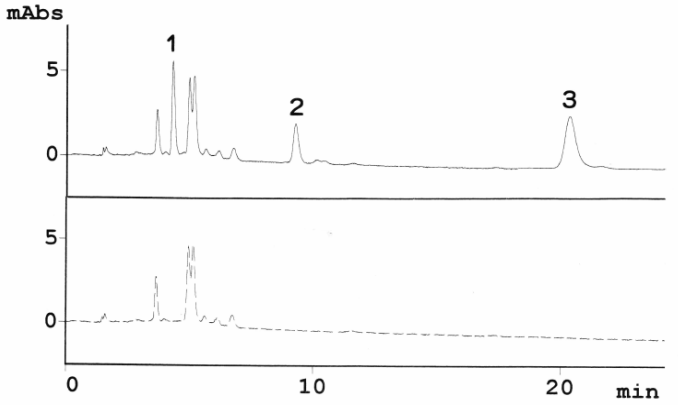


*Fig. 2.* Chromatograms obtained from the HPLC system for an egg yolk sample spiked with the target analytes (each 25 μg $g^{-1}$) (upper profile) and a blank egg yolk sample (lower profile). The PDA detector was set at 470 nm. Peaks, 1 = AX (retention time, $R_t$ = 4.29 min); 2 = CX ($R_t$ = 8.28 min); 3 = ACAEE ($R_t$ = 20.33 min)

*Table II*. The main method validation data

| Parameter | AX | CX | ACAEE |
|---|---|---|---|
| Linearity[a] | 0.998 | 0.995 | 0.989 |
| Accuracy[b] | 91.5–101.1 | 93.2–100.4 | 70.5–72.9 |
| Precision[c] | ≤2.9 | ≤2.8 | ≤5.0 |
| Sensitivity (QL[d]) | 0.3 | 0.5 | 1.0 |
| CC$\alpha$[e] | 0.09 | 31.19 | 0.3 |
| CC$\beta$[f] | 0.138 | 33.09 | 0.382 |

[a]*r* is the correlation coefficient (P < 0.01): mean of three determinations using spiked milk samples for calibration curves: ranges of concentrations were 1–50 μg $g^{-1}$ for all analytes.

[b]Average recoveries (%) from six replicates at three QC levels (5, 25, and 30 μg $g^{-1}$ for three analytes).

[c]Values are relative standard deviations (RSDs, %, $n$ = 6 of each level).

[d]Quantitation limit (μg $g^{-1}$), QL as the concentration of analyte giving a signal-to-noise ratio > 10.

[e]Decision limit ($\alpha$ = 5%, μg $g^{-1}$) by the EU regulation 2002/657/EC (as the established permitted limit = the EFSA's MRL 30 μg $g^{-1}$) [27].

[f]Detection capability ($\beta$ = 5%, μg $g^{-1}$) by the EU regulation 2007/657/EC [27].

## Method Validation

### Linearity and Range

The spiked recovery graph was generated as practical calibration line by plotting peak areas of fortified sample extracts ranging from 1 to 50 μg $g^{-1}$ versus their concentrations. The resulting line showed an excellent linearity for individual analyte.

### Accuracy and Precision

The average recoveries from egg yolk samples at three different QC levels (5, 25, and 30 μg $g^{-1}$ for three analytes) were 91.5–101.1% with relative standard deviations (RSDs) of 2.9 for AX and 93.2–100.4% with RSDs of ≤2.8 for CX, and 70.5–72.9% with RDSs of ≤5.0 for ACAEE, respectively. These values are well within the international method acceptance criteria [23, 25, 26].

### Sensitivity

The quantitation limit (QL), i.e., Sensitivity, should correspond to the concentrations for which the signal-to-noise ratio is less than 10. The QLs in egg yolk samples were 0.3 μg $g^{-1}$ for AX, 0.5 μg $g^{-1}$ for CX, and 1.0 μg $g^{-1}$ for ACAEE, respectively.

The decision limit (CC$\alpha$) and detection capability (CC$\beta$) values calculated according to the EU regulation decision (2002/657/EC) [28] are described in *Table II*. The above-mentioned findings are summarized in this table. The other validation findings are as follows:

### Specificity and Selectivity

The application of the proposed procedure to 10 blank egg yolk samples demonstrated that no interference peak was presented around the retention times for AX, CX, and ACAEE in any of the samples examined.

The present HPLC-PDA system easily confirmed the peak identity of target compound. All analytes were identified in an egg yolk sample by their retention times and absorption spectra. The AX, CX, and ACAEE spectra obtained from the egg yolk sample were practically identical to those of the standards. Because of the complete separations and the high absorbance of the analytes, PDA detection at trace levels is fully available. It is, therefore, instructive to demonstrate purification effectiveness of the sample preparation. The system did not require the use of MS, which is very expensive and is not available in a lot of laboratories for routine analysis.

### Robustness

**S**ome HPLC parameters were performed using a spiked (5 μg $g^{-1}$ of each compound) egg yolk sample obtained under the established procedure.

Changes of ±5% units of the flow rate (1.0 mL $min^{-1}$) and the column temperature (25°C) were determined. The effect on the peak areas and the validations in the retention times were evaluated. Changes of ±5% of the flow rate and the column temperature had no effect on the peak areas, whereas the variations in the retention times were obtained with the flow rate and the column temperature. Normal retention times for AX, CX, and ACAEE were 4.29, 8.28, and 20.33 min, respectively. At +5% the flow rate, the three retention times were decreased, ranging between 1.8% and 6.5% and at −5%, the times were increased ranging between 6.4% and 8.5%. By changing the column temperature by +5%, decreasing retention times obtained were 2.1–8.1%; however, no significant variations were observed with −5%. During these studies, all the target compounds were separated.

### System Suitability

The system suitability evaluation is an essential parameter of HPLC determination, and it ascertains the strictness of the system used. The suitability was evaluated as the relative standard deviations of peak areas and retention times calculated for 20 replicate injections of a spiked milk sample (25 μg $g^{-1}$ of each compound). The values for AX, CX, and ACAEE were estimated to be <0.5% for peak areas and <1.0% for retention times, respectively.

### Cost and Time Performance

The total time and budget required for the analysis of a single sample was <30 min and approximately EUR 4.0 (US $5.3) as of 19 February 2013, respectively. For sequential analyses, a batch of 24 samples could be analyzed in approximately 9 h. These findings became term required for the routine assay. The short analytical time not only increased the sample throughput for analysis but also positively affected the cost.

## Conclusions

A simplified sample preparation followed by HPLC-PDA method for simultaneous determination of AX, CX, and ACAEE, which are frequently used pigments to laying hens in egg yolks, has been successfully developed and

validated. The present procedure provided was easy-to-use, rapid, and space-saving and resulted in high recovery and repeatability with considerable saving of analysis time/cost. The procedure may be proposed as an international harmonized method for determining AX, CX, and ACAEE in the yolk of domestic/import eggs.

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## References

[1] European Food Safety Authority (EFSA), EFSA J., **291**, 1 (2005)
[2] EFSA, EFSA J., **507**, 1 (2007)
[3] The Natural Food Colour Association (published by webmaster on Fri, 04/29/2011), The legislation of food colours in Europe (http://www.natcol.org/node/5#13b)
[4] Japanese Ministry of Agriculture, Forestry and Fisheries law 40th (http://law.e-gov.go.jp/htmldata/S51/S51F00601000035.html)
[5] DSM Nutritional Products, DSM Guidelines for egg yolk pigmentation with CAROPHYLL® (http://www.christa.bg/files/Catalogue/105.pdf)
[6] Lonye Technology Limited (http://www.lonyetech.com/ProductsView.asp?id=136)
[7] J.C. Na, J.Y. Song, B.D. Lee, S.J. Lee, C.Y. Lee, and G.H. An, Anim. Feed Sci. Technol., **117**, 305 (2004)
[8] F. Sirri, N. Iaffaldano, G. Minelli, A. Meluzzi, M.P. Rosato, and A. Franchini, J. Appl. Poult. Res., **16**, 429 (2007)
[9] E. Santos-Bocanegra, X. Ospina-Osorio, and E.O. Oviedo-Rondón, Int. J. Poult. Sci., **3**, 685 (2004)
[10] Algae to Omega Holdings, Astaxanthin (http://www.algae2omega.com/ astaxanthin)
[11] Nichiwa Sangyo Co., Ltd., Patentjp.com. (http://www.patentjp.com/07/A/A100829/DA10002.html)
[12] Mercian Corporation, Patentjp.com. (http://www.patentjp.com/09/L/L100272/DA10001.html)
[13] Uehara Yokei (http://tamago-uehara.com/info.html)
[14] Astaxanthin (http://www.astaxanthine.info/2009/03/post_23.html)
[15] EFSA, EFSA J., **1852**, 1 (2010) (http://www.efsa.europa.eu/en/efsajournal/pub/1852.htm)
[16] EFSA, EFSA J., **1095**, 1 (2009) (http://www.efsa.europa.eu/en/efsajournal/pub/1098.htm)
[17] The Japan Food Chemical Research Foundation (JFCRF), 2009. (http://www.m5.ws001.squarestart.ne.jp/foundation/fooddtl.php?f_inq=19600)
[18] J. Schlatterer and D.E. Breithaupt, J. Agri. Food Chem., **54**, 2267 (2006)

[19] J. Rajesha, B. Madhusudhan, M.M. Swamy, R.J. Rao, G.A. Ravishankar, A. Rangarao, and M. Karunakumar, J. Food Sci. Technol. (Mysore), **46**, 509 (2009)
[20] M. Wenzel, I. Seuss-Baum, and E. Schlich, J. Agr. Food Chem., **58**, 1726 (2010)
[21] Furusawa, N., Food Chem., **124**, 1643 (2011)
[22] FDA/CDER/CVM, Guidelines for Industry—Bioanalytical Method Validation, 2001. (http://www.fda.gov/downloads/Drugs/GuidanceCompliance Regulatory-Information/Guidances/UCM070107.pdf)
[23] L. Huber, Validation and Quantification in Analytical Laboratories, Interpharm Press, East England, CO, USA, 1998
[24] ICH, Work Products, ICH Guidelines, Quality Guidelines (http://www.ich.org/products/guidelines.html)
[25] AOAC International, Method Validation, Guidelines & References (http://www.aoac.org/vmeth/guidelines.htm)
[26] Codex alimentarius commission, ALINORM 01/23, Codex Committee on Methods of Analysis and Sampling, Joint FAO/WHO Food Standards Programme, the 23rd Section of the Codex Committee on Method of Analysis and Sampling (http://www.codexalimentarius.net/web/archives.jsp?year=01)
[27] GL Sciences, Monolithic SPE Column for the Purification and Enrichment of Small Amount Sample, MonoSpin® Series (http://www.glsciences.com/products/monolithic_products/mono_spin.pdf)
[28] EU Decision 2002/657, Official Journal European Communication L221/8

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