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Determination of egg yolk xanthophylls by isocratic high-performance liquid chromatography

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ABSTRACT

An isocratic HPLC method was developed for the determination of eight xanthophylls (lutein, capsanthin, zeaxanthin, canthaxanthin, β-apo-8′-carotenal, ethyl-8′-apo-β-carotene-8′-oate, citranaxanthin and β-cryptoxanthin; registered as additives in poultry feeding) in egg yolks. Optimum separation of all-*E*-isomers of these xanthophylls was achieved in less than 18 min on a ProntoSIL C_{30} column at 27 °C using acetone–methanol–0.5 M triethylammonium acetate buffer pH 7 14:5:1 (v/v) as the mobile phase with a flow rate of 1 mL/min using spectrophotometric detection at 450 nm. Other mobile phases were also found suitable, including acetone–water 93:7 (v/v) and acetone–methanol 1:4 (v/v) and the influences of column temperature on the separation and addition of triethylammonium acetate buffer pH 7 to the mobile phase on enhancement of the peak areas were evaluated. Preparation of test solution from yolks included a short vortexing of 0.5 g of yolk in 10 mL of acetone, followed by 15 min magnetic stirring under nitrogen and centrifugation. The method was validated for 5 analytes. The calibration range was between 0.04 and 2 μg/mL and the mean recovery of the analytes (95%) and the intra-day precision of the method (RSD less than 5%) on three levels in triplicates were obtained. Analyses of eggs from four husbandry classes showed the presence of up to four xanthophylls (lutein, zeaxanthin, canthaxanthin and ethyl-8′-apo-β-carotene-8′-oate) and traces of β-cryptoxanthin.

1. Introduction

Eggs constitute basic food everywhere and in addition to many valuable micronutrients they also contain mixtures of carotenoids which, depending on content, give the yolk a more or less intense yellow-orange colouration. Carotenes have a low deposition efficiency in yolks [1,2] and the carotenoids in eggs are actually xanthophylls. Consumers typically prefer more intense colouration of yolks, and this is achieved naturally by organic farming which uses feed based on corn – a source of lutein, zeaxanthin and in smaller amounts β-cryptoxanthin – to which some other carotenoid-rich fodder, such as alfalfa may be added [3]. Since carotenoids also contribute to improved health of hens and chicken [4] they represent a desirable and even necessary part of the fodder. Currently, a majority of commercial eggs are produced by supplementing the fodder of hens with commercial carotenoid mixtures. This produces a higher carotenoid content than can be achieved by feeding with wheat, soy and barley, which contain lower levels of carotenoids than corn [4,5].

In the European Union eight xanthophylls are permitted in animal feed during egg production. As shown in Fig. 1, these are lutein (**1**, main source marigold – *Tagetes erecta* L.), zeaxanthin (**3**), β-cryptoxanthin (**8**), capsanthin (**2**, from paprika), canthaxanthin (**4**), β-apo-8′-carotenal (**5**), ethyl-8′-apo-β-carotene-8′-oate (**6**) and citranaxanthin (**7**) [5], the last four synthesised industrially and commercially available. The allowed maximum content of xanthophylls in poultry feed is 80 mg/kg with the exception of canthaxanthin, which content in poultry feed is limited to 8 mg/kg [3,5]. With respect to natural carotenoids, synthetic carotenoids have some improved properties such as colouration power, deposition efficiency and stability and can be less expensive than natural carotenoids [3,6]. Consumption of lutein and zeaxanthin is beneficial for human health, especially for prevention of age-related macular degeneration and cataracts [7,8]. A further important advantage is that the bioavailability of lutein from eggs is more pronounced than from spinach or food supplements [9].

The normal level of xanthophylls is 0.3–0.5 mg per yolk, a half of this is lutein, but under the influence of the enriched lutein diet of laying hens the lutein content can be 5–8 times higher [10]. Only carotenoids contribute to the yellow-orange colouration of yolks and spectrophotometric evaluation of yolk extract can give a rough estimate of total carotenoids [11]. Currently, HPLC is the main analytical technique used in carotenoid research but no standard

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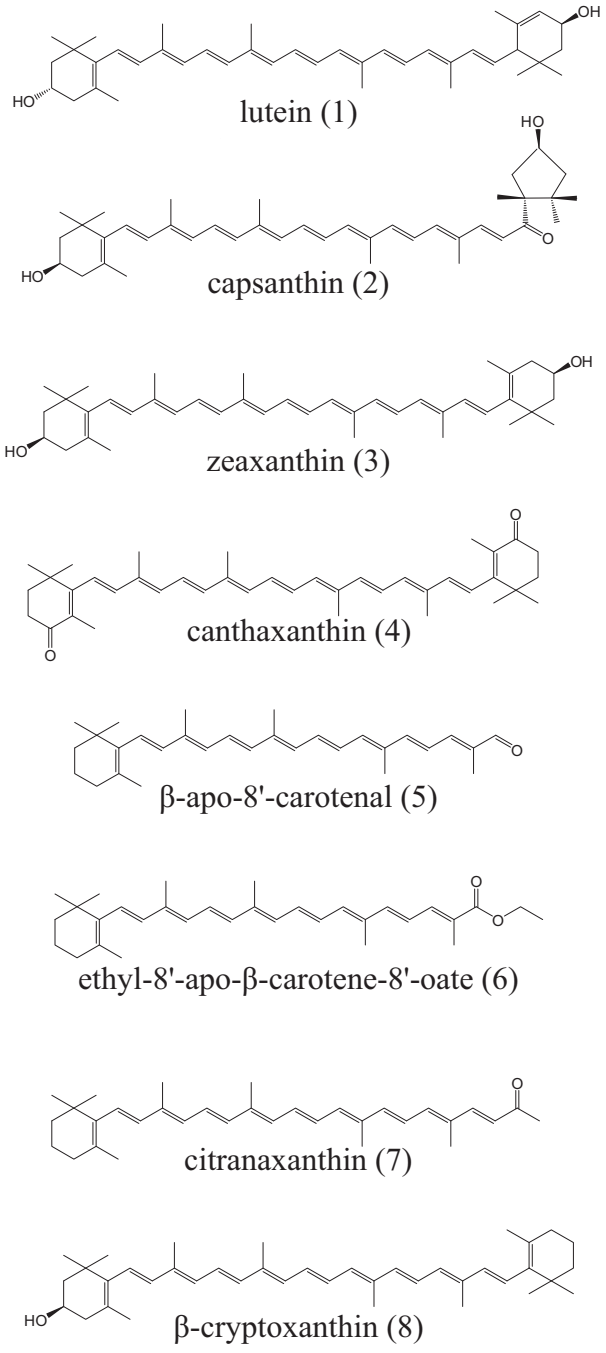

Fig. 1. The structures of 8 xanthophylls present in MIX8 and permitted as poultry feed additives.

method exists for determination of the eight carotenoids in eggs. Most reports have used UV–vis or photodiode-array (PDA) detectors for detection and determination of carotenoids because of their intense and specific absorption of visible light. Approaches used in preparation of yolk test solutions include one-phase [6,11,12], or two-phase extraction [4,13], extraction and elimination of lipids on a CN [14] or a silica mini-column [15], or saponification [1]. Chromatographic determination is performed most frequently on reversed-phase C18 [1,4,11,15–17] or C30 [6,9,13,18] columns with gradient elution, although a method using isocratic elution of four all-*E* yolk xanthophylls [6] was recently published. Among some 80 different HPLC–MS methods for determination and/or identification of carotenoids in different samples [19] only one reported identification and determination of carotenoids in yolks [13]. In spite of recent contributions achieved by HPLC–(APCI)MS [6,18] the analysis of geometric isomers and carotenoid metabolites in yolks remains incompletely described.

Table 1
Specific absorption coefficients ($A_{1\%,\,1\,cm,\,\lambda}$).

Compound	$A_{1\%,\,1\,cm,\lambda}$	λ_{max} (nm)	Solvent
Lutein[a]	2550	445	Ethanol
Capsanthin[b]	2076	469	*n*-Hexane/2% CH_2Cl_2
Zeaxanthin[a]	2540	450	Ethanol
Canthaxanthin[b]	2430	465	*n*-Hexane/2% CH_2Cl_2
β-Apo-8′-carotenal[b]	3069	455	*n*-Hexane/2% CH_2Cl_2
Citranaxanthin[b]	2868	467	*n*-Hexane/2% CH_2Cl_2
Ethyl-8′-apo-β-carotene-8′-oate[b]	2645	442	*n*-Hexane/2% CH_2Cl_2
β-Cryptoxanthin[b]	2578	450	*n*-Hexane/2% CH_2Cl_2

[a] Data from Ref. [20].
[b] Data from certificates for standards obtained from CaroteNature.

Natural or synthetic xanthophylls are added to hens' feed to achieve better quality yolks. For quality control and especially verification of organically produced eggs, the structure and quantity of the xanthophylls should be known and the goal of this study was development of an HPLC method for determination of the eight officially permitted xanthophylls as additives in poultry feed. The method uses a simple preparation of yolk test solution and chromatography on a C30 column and isocratic elution in optimised conditions with triethylammonium acetate buffer pH 7 as the additive in the mobile phase for enhancement of the peak areas. After successful validation of the most important analytical parameters the method was applied to the analysis of eggs from different husbandry classes.

2. Experimental

2.1. Chemicals

All chemicals were at least of analytical grade. Acetone (GC grade) and 1 M triethylammonium acetate (TEAA) were purchased from Sigma–Aldrich Chemie (Steinheim, Switzerland), methanol (HPLC grade) from J.T. Baker (Deventer, the Netherlands), and chloroform from Merck (Darmstadt, Germany). Standards of β-apo-8′-carotenal (HPLC 97%), canthaxanthin (HPLC 98%), capsanthin (HPLC 96%), citranaxanthin (HPLC 98%), β-cryptoxanthin (HPLC 97%), lutein (HPLC 96%), zeaxanthin (HPLC 97%), ethyl-8′-apo-β-carotene-8′-oate (HPLC 96%) were obtained from CaroteNature (Lupsingen, Switzerland). For validation, standards of lutein (UV–vis ≥ 95%), zeaxanthin (UV–vis ≥ 98%), β-cryptoxanthin (UV–vis ≥ 97%) were purchased from Extrasynthèse (Genay Cedex, France), while canthaxanthin (HPLC 94%) and β-apo-8′-carotenal (UV–vis ≥ 96%) were from Sigma–Aldrich (St. Louis, Missouri, USA). All-*E*-isomers were present almost exclusively in all standards and all the experiments and determinations in eggs refers only to these isomers. Water was bidistilled.

2.2. Preparation of standard solutions

For preparation of standard stock solutions (20 μg/mL) a few drops of chloroform was used to facilitate the dissolution of carotenoid standards in acetone previously purged with nitrogen. The precise concentration of each xanthophyll in the standard solutions was determined spectrophotometrically using the known specific coefficient of maximum absorption at the appropriate wavelength and solvent of the corresponding xanthophyll (data from [20] and from certificates for standards obtained from CaroteNature, Table 1). The chromatographic purity of each xanthophyll at 450 nm (isocratic mode, mobile phase: acetone–methanol–0.5 M