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Macular carotenoids in lipid food matrices: DOE-based high energy extraction of egg yolk xanthophylls and quantification through a validated APCI(+) LC-MS/MS method

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ABSTRACT

Lutein and zeaxanthin exhibit significant biological activities therefore their dietary intake through carotenoid-rich foods and supplements is strongly recommended as preventive approach. Hence their extraction from natural substrates targets to their commercial exploitation as nutraceuticals and ocular pharmaceuticals. Since carotenoids' bioavailability is higher in fat-containing substrates, egg yolk is considered an ideal food matrix. DOE-based optimization of novel high energy extraction practices achieves efficient recovery of xanthophylls from lipid sources. In this research, 2^3 full factorial and Box-Behnken designs (BBD) were applied for optimizing ultrasound- (UAE) and microwave-assisted extraction (MAE) variables (i.e. extraction solvent, temperature, time, US or MW power and solvent/material ratio). LC-MS/MS results pointed out the precedence of UAE in lutein and zeaxanthin extraction, where higher yields were obtained with 1:1 n-hexane-acetone as solvent mixture at 19 min, 600 W and 35 mL g^{-1}. UAE carotenoid content was higher than MAE due to the different mechanisms laying behind the two processes and due to more complete granule rupture caused by higher US power. Evaluating the current results, DOE-based UAE analytical methodology stands out as an auspicious and sustainable alternative for commercial-based extraction of lipidic bioactive compounds for food and drug industrial applications.

1. Introduction

Lutein and zeaxanthin, two lipid-soluble alcohol conjugated dienes with eight isoprenoid units, which belong to xanthophylls' carotenoid group, are major natural colorants of plants (maize, saffron) and of animal products (marine organisms, avian eggs). Since animals are not capable of carotenoids biosynthesis, it is necessary to receive them from their diet [1].

The health promoting effects of xanthophylls' carotenoids and especially lutein and zeaxanthin, have been well established mainly due to their antiiflammatory [2] and antioxidant properties which enables their chemoprotective action and beneficial activity against cardiovascular and Alzheimer's disease [3]. Furthermore, these carotenoid isomers are important bioactive ingredients in novel skinceutical preparations with confirmed positive effects on skin hydration and elasticity [4]. Recent studies revealed also a relation between macular xanthophylls and increased bone mass in young adults [5].

Interestingly, this group of biomolecules has a great impact on ocular tissues therefore, the term “macular carotenoids” has been assigned to them. As stated in 2014 report of Centers for Disease Control and Prevention (CDC), the estimated number of people affected by ocular impairments (i.e. age-related macular degeneration (AMD) and cataract) worldwide will be doubled by 2030 [6]. Recent clinical trials proved that lutein and zeaxanthin-enriched supplements and diet could mitigate or even prevent the risk of retinopathies, which affect over 40% of worldwide population and bear down on financial indices and policies [7].Conforming to nutritional and medical surveys (i.e. National Health and Nutrition Examination Survey) an individual

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suffering from eye impairments needs an average dose of 6–40 mg of lutein per day depending on the type and severity of vision damage, while a person with an ordinary diet receives daily a lutein amount of almost only 2 mg [8].

Normally lutein content in dietary sources is five times higher than zeaxanthin. In foods containing fats and lipids, xanthophylls and their esters are present in lower yields, but they show higher bioavailability than carotenoids of plant origin (e.g. spinach and kale) [9]. In that case, higher bioaccessibility of carotenoids is attributed to the lipid nature of the dietary source since lipids and fats enable carotenoids' solubilization and eventually dispersion in human digestive fluid, intestinal absorption and finally retinal accumulation [10]. According to scientific findings, lutein eye concentration reaches an increase of over 20% in cases where egg consumption is daily for 3 months period [11]. Thus, current drug formulations contain lutein encapsulated in solid lipid nanocarriers, phospholipid suspensions and nanostructure lipid carriers [12,13].

Relying on the preventive and therapeutic effects of various of its micro- and macro- nutrients (lipids, carotenoids, proteins, minerals and vitamins) eggs have overcome the negative reputation of 'health-risk food', related to its high cholesterol content, and they have won an important position in daily human diet [9]. Furthermore, choline, betaine and ω-3 fatty acids are metabolites related to the protection from inflammations, allergies, heart diseases, strokes, arthritis and cataract, while egg yolk proteins inhibit human platelet aggregation [14]. Thus, egg yolk could be designated as the ideal candidate for studies regarding lutein delivery and absorption in humans.

Nowadays, high energy techniques fulfill the purposes of environmental-friendly large-scale extraction approaches for the recovery of bioactive targets from natural sources and by-products. The advantageous points of UAE and MAE techniques [15] are summarized to: (a) increased extraction yields in shorter extraction time, (b) use of alternative eco-friendly solvents and reduced amounts of hazardous derivatives, (c) energy- and time-saving procedures, (d) downscaling of operational units in industrial processes through new set-ups and technologies, (e) protection of thermolabile biomolecules and (f) enhancement of extraction selectivity for valuable compound classes (lipids, polyphenols and phenolic compounds, flavonoids and anthocyanins, polysaccharides and carotenoids) [15].

High energy techniques could be considered as feasible extraction alternatives only through determining their critical operational parameters and their optimal values by performing the minimum necessary number of experiments. Experimental design (DOE) is applied for this purpose including screening of experimental factors as the first step of DOE strategy. Discovering exact optimal conditions using response surface methodology (RSM) follows. Optimal conditions can be predicted by a quadratic term-equation applying three- or more level-designs, such as central composite and Box-Behnken designs [15].

New trends in pharma- and nutra-companies steer current research towards the study of natural lipid substrates which act as carriers of important bioactive compounds [12,13]. Due to its unique health-promoting functions and its high lipidic content egg yolk emerges as the most adequate lipid substrate for reviewing xanthophylls delivery, absorbance and interaction with lipid constituents. Therefore, the development of a validated modern analytical methodology is of pivotal importance. A DOE-based high energy extraction - LC-MS/MS new analytical procedure fits this purpose as it will open the road for cutting edge nutricosmetics, cosmeceuticals and nutraceutical innovations (dermal products, food supplements, eye drops, capsules and sprays) [16,17]. Hence, our study is aiming to examine lipidic effect of natural components on carotenoids extraction and determination. In addition, our purpose is to introduce an in-house analytical methodology by merging high throughput analytical tools of (a) DOE-oriented high energy extraction and (b) APCI(+) LC-MS/MS fingerprint, for future scaling-up of xanthophylls recovery from fat rich food matrices, lipid food byproducts, lipid drug systems and formulations.

2. Materials and methods

2.1. Reagents and chemicals

trans-Lutein and *trans*-zeaxanthin were obtained from Extrasynthese (Genay, France). *trans*-Canthaxanthin was acquired from Fluka (St. Louis, USA) and *trans*-β-apo-8′-carotenal from Sigma-Aldrich (St. Louis, USA). All solvents tested were of analytical grade. Methanol, chloroform and *n*-hexane were purchased from Merck (Darmstadt, Germany) while acetone from ChemLab (Zedelgem, Belgium). LC-MS grade methanol, acetonitrile and methyl-*tert*-butyl ether (MTBE) were acquired from Scharlau (Barcelona, Spain), Fischer Chemical (Pittsburgh, USA) and Fluka (Darmstadt, Germany).

2.2. Sample treatment and preparation

Eggs were kindly provided by the department of Animal Breeding and Husbandry in the faculty of Animal Science and Aquaculture of the Agricultural University of Athens from hens of subspecies *Gallus gallus domesticus* of Phasianidae family [18]. Egg yolks (n = 23) were separated *per manus* from eggs and were freeze dried in a ModulyoD Freeze Dryer, equipped with a Thermo Savant ValuPump VLP200 (Thermo Electron Corporation, Thermo Fischer, USA). Then dried material was homogenized and powdered in a laboratory mill (Type ZM1, Retsch GmbH, Haan, Germany). Dry powdered material, UAE and MAE extracts and LC-MS standard solutions and samples were kept in glass jars and vials at −20 °C.

2.3. Extraction apparatuses and instruments

Ultrasound-assisted extraction (UAE) process was conducted by a Vibra-Cell VCX 750 (20 kHz, 750 W) ultrasonics processor (Sonics and Materials Inc., Newtown, USA), equipped with piezoelectric converter and 13 mm diameter probe fabricated from titanium alloy Ti–6Al–4 V. A Centrifuge CL30 (Thermo Scientific, UK) was used for the classical extraction. Microwave-assisted extraction (MAE) process was conducted using a CEM Focused Microwave System, Model Discover (CEM Corporation, Matthews, USA), in open vessel or focused microwave (FMAE) mode with a reflux system placed above the open cell.

2.4. Extraction procedures

Three grams (3 g), for UAE, and 1 g (1 g), for MAE, of dried egg yolk powder was suspended in several volumes of different extraction solvents. In detail, pure chloroform, methanol, n-hexane, acetone and mixtures of various combinations of the aforementioned solvents were used.

For classical extraction, the procedure was performed with 12:1 (v/w) solvent/material ratio and according to a modified 3-step Folch method [19]. Briefly, 1 g of dried yolk powder was homogenized with 6 mL of 2:1 chloroform-methanol (v/v) and it was vortexed thoroughly. The mixture was allowed to equilibrate for 10 min, and the supernatant was collected after centrifugation at 3000 rpm for 5 min. The residue was re-extracted twice with 3 mL of the Folch solvent mixture following the procedure as described above. Thereafter, the recovered extracts were pooled and 2.4 mL of water was added to form a two-phase solvent system, which was left to equilibrate overnight at room temperature. The lower chloroformic phase was recovered, while the polar water-methanol phase was discarded.

After each extraction process, the solid material was separated from the supernatant under vacuum filtering and the extract was evaporated for solvent removal using a rotary evaporator at 50 °C [20]. Next, the lipid fraction of the extract was diluted in 8 mL of extraction solvent and a volume aliquot until 100 mg of carotenoid extract was flushed with N_2 stream. Then, the dry residue was dissolved in 10 mL of acetone and the total carotenoid content was estimated by the