Food Chemistry 163 (2014) 147–153

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Tackling the challenge of selective analytical clean-up of complex natural extracts: The curious case of chlorophyll removal

Sebastiaan Bijttebier [a,b,*], Els D'Hondt [a], Bart Noten [a], Nina Hermans [b], Sandra Apers [b], Stefan Voorspoels [a]

[a] Flemish Institute for Technological Research (VITO), Business Unit Separation and Conversion Technology (SCT), Boeretang 200, 2400 Mol, Belgium
[b] University of Antwerp, NatuRA, Universiteitsplein 1, 2610 Antwerp, Belgium

ARTICLE INFO

Article history:
Received 4 February 2014
Received in revised form 25 April 2014
Accepted 27 April 2014
Available online 9 May 2014

Chemical compounds studied in this article:
Chlorophyll *a* (PubChem CID: 6477652)
Triacylglycerol (16:0/16:1/16:1) (PubChem CID: 9543987)
Diacylglycerol (16:0/16:1) (PubChem CID: 9543678)
Fucoxanthin (PubChem CID: 5281239)
trans-β-Apo-8′-carotenal (PubChem CID: 5478003)
Citranaxanthin (PubChem CID: 6436724)

Keywords:
LC-PDA–am-MS
Saponification
Ambersep 900 OH
Selective chlorophyll removal mechanism
Carotenoids
High valuable oils

ABSTRACT

Alkaline saponification is often used to remove interfering chlorophylls and lipids during carotenoids analysis. However, saponification also hydrolyses esterified carotenoids and is known to induce artifacts. To avoid carotenoid artifact formation during saponification, Larsen and Christensen (2005) developed a gentler and simpler analytical clean-up procedure involving the use of a strong basic resin (Ambersep 900 OH). They hypothesised a saponification mechanism based on their Liquid Chromatography-Photodiode Array (LC-PDA) data. In the present study, we show with LC-PDA–accurate mass-Mass Spectrometry that the main chlorophyll removal mechanism is not based on saponification, apolar adsorption or anion exchange, but most probably an adsorption mechanism caused by H-bonds and dipole–dipole interactions. We showed experimentally that esterified carotenoids and glycerolipids were not removed, indicating a much more selective mechanism than initially hypothesised. This opens new research opportunities towards a much wider scope of applications (e.g. the refinement of oils rich in phytochemical content).

1. Introduction

Carotenoids consist of a large group of apolar natural compounds that are predominantly found in plants, algae and bacteria (Britton, Liaaen-Jensen, & Pfander, 1994). For several decades, carotenoids have been produced commercially by chemical synthesis or as plant extracts or oleoresins to be used as colorants and food supplements (Britton, Liaaen-Jensen, & Pfander, 2009). Carotenoids play a key role in photosynthesis (Armstrong & Hearst, 1996) and are identified as potentially important natural compounds that might aid in the prevention of several human chronic degenerative diseases, such as cancer, cardiovascular diseases and age-related eye diseases (Agarwal, Parameswari, Vasanthi, & Das, 2012; Chatterjee, Roy, Janarthan, Das, & Chatterjee, 2012; Ilic & Misso, 2012; Ozawa et al., 2012; Riccioni, 2009; Su, Rowley, & Balazs, 2002; Tanaka, Shnimizu, & Moriwaki, 2012; Wojcik, Burzynska-Pedziwiatr, & Wozniak, 2010). This urges the need for analytical methods that enhance rapid identification of carotenoids in different matrices to identify the various natural sources of these valuable nutrients.

Identification of carotenoids with spectrophotometric detection can be hampered by co-elution of high chlorophyll concentrations. Classically, an alkaline saponification procedure is used to remove chlorophylls (Britton et al., 1994). However, alkali treatment may cause isomerization and several other unwanted reactions of carotenoids (aldol condensation, dehydration, retro-aldol cleavage and other alkali reactions) (Britton et al., 1994). Furthermore, alkaline saponification is not selective and hydrolyses ester bonds of other plant metabolites such as lipids and carotenoids acylated

* Corresponding author at: Flemish Institute for Technological Research (VITO), Business Unit Separation and Conversion Technology (SCT), Boeretang 200, 2400 Mol, Belgium. Tel.: +32 14 33 50 54; fax: +32 14 31 94 72.
E-mail addresses: sebastiaan.bijttebier@vito.be (S. Bijttebier), els.dhondt@vito.be (E. D'Hondt), bart.noten@vito.be (B. Noten), nina.hermans@uantwerpen.be (N. Hermans), sandra.apers@uantwerpen.be (S. Apers), stefan.voorspoels@vito.be (S. Voorspoels).

http://dx.doi.org/10.1016/j.foodchem.2014.04.098
0308-8146/

with fatty acids (Britton et al., 1994). Such treatment therefore results in loss of the natural composition of the sample.

To avoid carotenoids losses during saponification, Larsen and Christensen (2005) developed a gentler and simpler analytical clean-up procedure involving the usage of a strong basic quaternary amine styrene–divinylbenzene anion resin (Ambersep 900 OH). From the results obtained by Liquid Chromatography-Photodiode Array (LC-PDA) analysis, they hypothesised that carotenoid esters, chlorophylls and other compounds containing ester bonds such as lipids were saponified during the clean-up (Larsen & Christensen, 2005). Although they demonstrated the removal of chlorophylls experimentally, they did not confirm that lipids and carotenoid esters were hydrolyzed. Hence, anion exchange or adsorption could also be the main mechanism of chlorophyll removal by the Ambersep 900 OH resin. An anion exchange or adsorption based on interionic and intermolecular forces (e.g. dipole–dipole interactions, hydrogen bonding) would imply a more selective clean-up without the formation of artifacts produced by saponification (e.g. hydrolysis of ester bonds, etc.). It has been described that compounds generated by light-induced degradation of chlorophylls can contribute significantly to an oxidised odour and off-flavors (Wold et al., 2005). Acid activated bleaching clays have been used extensively for the large-scale discoloration of oils, however, carotenoids are also removed (Hussin, Aroua, & Daud, 2011; Sabah, Çinar, & Celik, 2007). The selective removal of chlorophylls from oils with retention of bioactive carotenoids and other phytochemicals could lead to new niche products such as algal oil based cosmetics intermediates and functional foods.

Mass Spectrometry (MS) detectors enable the simultaneous detection of a wide range of plant metabolites (Zhang et al., 2013). This is for instance very useful in unraveling the molecular constituents of algae producing both high concentrations of lipids and carotenoids (Nobre et al., 2013). Lipids from algae are often rich in the high valuable long-chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and are a potential sustainable source of these fatty acids for use in food or feed (Ryckebosch, Muylaert, & Foubert, 2012). Furthermore, MS detectors are much more selective than PDA detectors and can provide more structural information. Accurate mass (am) MS instrumentation such as Orbitrap MS detectors enable the tentative identification of unknown compounds based on the most probable molecular formulae that can be calculated from the detected precursor and product ions (Kind & Fiehn, 2007).

The objective of this work was to examine the applicability of the strong basic Ambersep 900 OH resin for selective chlorophyll removal from natural extracts with a high phytochemical content. The mechanism of action was examined by LC-PDA–am-MS analysis to determine its selectivity and to study the formation of phytochemical artifacts (e.g. hydrolysis of carotenoid esters and glycerolipids). Carotenoids and other plant metabolites were examined in sample extracts before and after clean-up with the Ambersep 900 OH resin to clarify if the chlorophyll removal is due to alkaline saponification, adsorption or an anion exchange mechanism. Proof of the feasibility of this clean-up procedure for the selective removal of chlorophylls and their derivatives on analytical scale would open new research opportunities towards a much wider scope of applications (e.g. the refinement of oils rich in phytochemical content).

2. Materials and methods

2.1. Chemicals

LC/MS-grade methanol, acetonitrile and ethyl acetate were purchased from Biosolve (Valkenswaard, The Netherlands). Ultrapure water with a resistivity of 18.2 MΩ·cm at 25 °C was generated with a Millipore system. Dichloromethane (DCM) for gas chromatography, *n*-hexane for gas chromatography, acetone for gas chromatography, sodium hydrogen carbonate, sodium hydroxide and sodium chloride for analysis were purchased from Merck (Darmstadt, Germany). Ammonium acetate, (D-Ala^2)-leucin enkephalin, sand (quartz), tetraethylammonium hydroxide, Ambersep 900 OH (20–50 mesh), Amberlite XAD-4 (20–60 mesh) and butylated hydroxytoluene (BHT) were purchased from Sigma–Aldrich (Bornem, Belgium). Ambersep 900 OH is a strong basic resin, which is composed of approximately 35–55% quaternary amine styrene–divinylbenzene co-polymer of the OH form and 45–65% water (Larsen & Christensen, 2005). Amberlite XAD-4 is a hydrophobic styrene–divinylbenzene co-polymer.

Lutein, zeaxanthin and violaxanthin were purchased from Carotenature (Ostermundigen, Switzerland). β-Carotene and *trans*-β-Apo-8′-carotenal were purchased from Sigma–Aldrich (Bornem, Belgium). Glycerol dioleate and glycerol trioleate were obtained from Chem Service (West Chester, PA).

The freeze dried algae *Isochrysis galbana* was kindly provided by the research group Food & Lipids from the Catholic University of Leuven campus KULAK (Kortrijk, Belgium), that obtained the algal biomass from Algaenergy (Madrid, Spain).

2.2. Preparation of standard solutions

Standard stock solutions and working solutions were prepared of each analyte separately at a concentration of approximately 200 μg/mL. Stock solutions of glycerolipids were prepared in methanol. Stock solutions of carotenoids were prepared in DCM + 0.1% BHT. Standard stock and working solutions were stored at −25 °C in the dark under an inert atmosphere (nitrogen). Dilutions of the stock solutions were prepared in DCM + 0.1% BHT for analysis.

2.3. Sample preparation

A method previously developed for the extraction of a wide array of apolar plant metabolites such as carotenoids and lipids was applied for extraction (Bijttebier et al., 2014). Approximately 1 g of freeze dried algae was spiked with *trans*-β-Apo-8′-carotenal (internal standard). The sample was subsequently mixed with approximately 1 g of sodium hydrogen carbonate and sand. Ultrapure water was added until the sample was hydrated (approximately 3 mL) and was let to rest in the dark under N_2 for 30 min to allow swelling of the matrix for better analyte extraction. Afterwards, the mixture was homogenised with sand and loaded into a 33 mL Accelerated Solvent Extraction (ASE) cell (Thermo Fisher Scientific, Bremen, Germany). The mixture was extracted 3 times with 70:30 acetone:methanol + 0.1% BHT (*v*:*v*) at 40 °C and 1050 psi. The three extracts were combined in a separating funnel and 100 mL 10% NaCl (aqueous) and 15 mL hexane was added. The hexane phase was transferred to a recipient after vigorous shaking and the polar phase was extracted twice more with 15 mL hexane. The combined hexane fractions were evaporated to dryness, dissolved in 10 mL DCM + 0.1% BHT and stored in the dark under nitrogen at −25 °C until further analysis.

For chlorophyll removal with the strong basic resin, the procedure according to Larsen and Christensen (2005) was used. Briefly, 0.5 mL of algal DCM extract was evaporated and redissolved in 5 mL of acetone. Approximately 0.5 g of Ambersep 900 OH resin was added and the mixture was shaken for 30 min. Afterwards, the acetone extract was transferred and the resin was washed 3 times with 5 mL acetone. The combined acetone extracts were evaporated, redissolved in 0.5 mL DCM and analysed within 12 h after extraction. To ensure repeatability of results, 5 replicates of the chlorophyll removal procedure were produced. The recoveries