**NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS**

**SCHOOL OF SCIENCE**

**DEPARTMENT OF CHEMISTRY**

**PhD THESIS**

# «Development and Validation of LC-MS and NMR methods for the Qualitative and Quantitative Determination of Carotenoids in Food and Byproducts»

**THALIA TSIAKA**
**CHEMIST, MSc**

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# «Ανάπτυξη και επικύρωση μεθόδων LC-MS και NMR για τον ποιοτικό και ποσοτικό προσδιορισμό καροτενοειδών σε τρόφιμα και παραπροϊόντα».

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# ABSTRACT

In recent years, consumers' changing preferences created a new flourishing economy based on products containing natural extracts with health-promoting properties. Furthermore, as food-industry waste turns into a global concern, the valorization of bioactive compounds from byproducts of plant or animal origin is an urgent need. In this thesis, we studied the recovery of carotenoids from byproducts of plant (apricots) and animal (shrimp head) natural substrates and lipid food matrices (egg yolk and shrimp body) by implementing an integrated analytical platform including (a) experimental design (DOE)-based high energy extraction processes using conventional and innovative green extraction solvents (natural deep eutectic solvents, NADES), (b) high throughput analytical techniques (Liquid Chromatography-Mass Spectrometry, LC-MS/MS and Nuclear Magnetic Resonance spectroscopy, NMR) and (c) multivariate unsupervised (PCA) and supervised (OPLS-DA) statistical analysis. In particular, ultrasound-assisted (UAE) and microwave-assisted extraction (MAE) were applied for the recovery of carotenoids from the examined substrates. The optimization of extraction conditions was carried out by using a two-level screening ($2^3$ full factorial) model and a response surface methodology (RSM) model (Box-Behnken design). A LC-MS/MS method was developed for the quantitation of carotenoids in order to determine the most efficient extraction approach for each substrate. Moreover, 1D- and 2D-NMR spectroscopy were applied to obtain the metabolic fingerprint of apricot byproducts' extracts in response to extraction techniques and conditions. PCA and OPLS-DA models allowed the classification of apricots extracts and provided information about co-extracted secondary metabolites. To sum up, the current thesis provide a proof-of-concept for the future large-scale implementation of the developed analytical platform for the conversion of natural byproducts to high-added value nutraceuticals and for the commercial exploitation of lipid foods of high bioactive content.



**SUBJECT AREA**: High energy extraction methodologies

**KEYWORDS**: Carotenoids, High energy extractions, Experimental design; NMR spectroscopy, Liquid chromatography-mass spectrometry

# ΠΕΡΙΛΗΨΗ

Τα τελευταία χρόνια, οι νέες καταναλωτικές συνήθειες έδωσαν ώθηση στην αγορά φυσικών προϊόντων με ευεργετικές ιδιότητες. Επιπρόσθετα, η αύξηση των φυσικών παραπροϊόντων καθιστά ως επιτακτική ανάγκη την αξιοποίησή τους μέσω της παραλαβής βιοδραστικών ενώσεων από αυτά. Στην παρούσα διατριβή, μελετήθηκε η ανάκτηση καροτενοειδών από παραπροϊόντα φυτικής (βερίκοκα) ή ζωϊκής (κεφάλι γαρίδας) προέλευσης και από λιπιδικά τρόφιμα (κρόκος αυγών και σώμα γαρίδας) εφαρμόζοντας μια συνδυαστική αναλυτική προσέγγιση που περιλαμβάνει (α) εκχυλίσεις υψηλών ενεργειών με χρήση συμβατικών και καινοτόμων πράσινων διαλυτών (φυσικοί βαθιά ευτηκτικοί διαλύτες, NADES) και πειραματικού σχεδιασμού (DOE) (β) υγροχρωματογραφία συζευγμένη με φασματομετρία μάζας (LC-MS/MS) και φασματοσκοπία πυρηνικού μαγνητικού συντονισμού (NMR) και (γ) μοντέλων πολυμεταβλητής στατιστικής ανάλυσης. Πιο συγκεκριμένα, εφαρμόστηκε εκχύλιση με υπερήχους (UAE) και με μικροκύματα (MAE) για την παραλάβη καροτενοειδών από τα μελετούμενα υποστρώματα. Οι συνθήκες εκχύλισης βελτιστοποιήθηκαν με δυο διαφορετικά DOE μοντέλα ($2^3$ full factorial design, Box-Behnken design). Η LC-MS/MS ποσοτικοποίηση των καροτενοειδών υπέδειξε την καταλληλότερη μεθόδο εκχύλισης για κάθε υπόστρωμα. Τα φάσματα NMR (1D-, 2D-NMR) ανέδειξαν το μεταβολικό αποτύπωμα των εκχυλισμάτων παραπροϊόντων βερίκοκου συναρτήσει των τεχνικών και συνθηκών εκχύλισης. Η ταυτοποίηση των συνεκχυλιζόμενων μεταβολιτών υπεύθυνων για την ταξινόμηση των εκχυλισμάτων και η αξιολόγηση της επίδραση των συνθηκών εκχύλισης σε αυτούς πραγματοποιήθηκε μέσω μοντέλων πολυμεταβλητής στατιστικής ανάλυσης (PCA, OPLS-DA). Συνοψίζοντας, η μελλοντική πιλοτική εφαρμογή της παρούσας αναλυτικής μεθοδολογίας μπορεί να οδηγήσει στην μετατροπή των παραπροϊόντων σε προϊόντα υψηλής προστιθέμενης αξίας και στην εμπορική αξιοποίησή τους σε διάφορους τομείς της βιομηχανίας (τρόφιμα, φάρμακα, καλλυντικά, τροφοφάρμακα).

**ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ**: Τεχνικές εκχύλισης υψηλών ενεργειών

**ΛΕΞΕΙΣ-ΚΛΕΙΔΙΑ**: Καροτενοειδή; Εκχυλίσεις υψηλών ενεργειών; Πειραματικός σχεδιασμός; Φασματοσκοπία NMR; Υγρόχρωματογραφία-φασματομετρία μάζας

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# CONTENTS

# FIGURE LIST

# TABLE LIST

# INTRODUCTION

The present PhD thesis was conducted from February 2013 to April 2019 at the Institute of Chemical Biology (ICB) and former Institute of Biology, Medicinal Chemistry and Biotechnology (IBMCB) in National Hellenic Research Foundation (NHRF).

Specifically, high energy extraction experiments were carried out at the laboratory of Organic and Organometallic Chemistry of ICB in NHRF. LC-MS/MS and NMR experiments were performed at the laboratory of Molecular Analysis of ICB in NHRF. Classic extractions and *vis*-spectrophotometric measurements were conducted at the laboratory of Chemistry, Analysis & Design of Food Processes of the Department of Food Science and Technology in University of West Attica. Natural deep eutectic solvents (NADES) were synthesized and provided by the group of Associate Professor Anastasia Detsi of the Laboratory of Organic Chemistry at School of Chemical Engineering of National Technical University of Athens.

Apricots' byproducts samples of this study were provided by Danais S.A Fruit Processing Industry & Export Company (www.danais-sa.com) (Argos, Peloponnese, Greece). Egg yolk samples were provided by Michael Goliomytis, Lecturer, Panagiotis E. Simitzis, Agricultural Researcher, Maria Charismiadou, Assistant Professor and Stelios G. Deligeorgis, Professor from the Agricultural University of Athens, Department of Animal Breeding and Husbandry in the faculty of Animal Science and Aquaculture.

In particular, the current thesis consists of the following chapters:

Chapter 1 is an introduction to the field of natural products obtained by agro- or seafood byproducts or lipid foods. In this chapter, the importance of high-added value byproducts and of foods of high bioactive content is discussed, focusing on apricot and shrimp byproducts, egg yolk and shrimp body.

Chapter 2 is dedicated to carotenoids, the target compounds of this thesis. Particularly, we provide information regarding their chemistry, bioactivity, occurrence and bioavailability. Special mention is given to the carotenoids of apricot byproducts, egg yolk and shrimps, i.e b-carotene, lutein, zeaxanthin, astaxanthin and canthaxanthin.

Chapter 3 provides a thorough overview of innovative non-conventional modern extraction approaches, like high energy extraction techniques. Especially, the chapter is we are focused on the summary of ultrasound- and microwave-assisted extraction and their current advances, of the solvents used in these techniques (conventional and green

solvents (natural deep eutectic solvents)) and of the coupling of high energy extraction with experimental design strategies (DOE) for the recovery of carotenoids from the investigated substrates.

The aim and the main objectives of the thesis are described in Chapter 4.

All materials and analytical methods used in this study are reported in detail in Chapter 5. Thorough information is provided for the implementation of DOE-based UAE and MAE techniques and the *vis*-estimation of extracts carotenoids content, for the synthesis and characterization of natural deep eutectic models (NADES), for the liquid chromatography-mass spectrometry (LC-MS/MS) analysis of carotenoids from all examined substrates and for the nuclear magnetic resonance (NMR) spectroscopy for acquiring the metabolic fingerprint of apricots DOE-extracts.

Chapter 6 includes the implementation of UAE and MAE coupled with DOE models for evaluating the effect of extraction parameters (extraction solvent, temperature, time, US or MW power and solvent/material ratio) on carotenoid content of apricot pulp, egg yolk and shrimp head and body and the optimization of extraction procedures for each substrate using $2^3$ full factorial and Box-Behnken designs. A comparative study between conventional organic solvent and NADES is also presented.

The development and validation steps of the LC-PDA-MS/MS method used for the quantitation of matrix carotenoids and the identification of cis-isomers of the substrates are presented in Chapter 7.

The metabolic fingerprint of apricot pulp extracts, the classification of DOE-extracts and the elucidation of key co-extractants by implementing 1D- and 2D-NMR spectroscopy and multivariate analysis is discussed in Chapter 8.

In conclusion, the goal of the current study is to develop an integrated and combinatorial analytical platform as a basic research robust tool and to provide a proof-of-concept for the further valorization of natural byproducts and foods by designating the importance of high energy extraction techniques and eco-compatible solvents.

# CHAPTER 1
# NATURAL PRODUCTS AND BYPRODUCTS

## 1.1 Natural products and byproducts: The pharmacy of nature

Rephrasing the famous quote of Hippocrates, “Let food be thy medicine and medicine be thy food,” modern scientists could state that natural products, either of plant, microbial, or animal origin, could not only be the food but also the medicine of present and future societies. Following the example of ancient world physicians and pharmacy practitioners, nowadays cosmetic and nutraceutical companies have focused their research to the production and launching of active molecules from natural sources [1].

Over the last 20 years, natural products and their extracts play a key role in almost every field of chemistry and biology, from microbiology and biochemistry to medicine and bioinformatics. In addition, consumers’ changing preferences have created a new flourishing economy based on products containing natural extracts with renowned health-promoting properties. Based on World Health Organization (WHO) estimations, a large part of world population still relies on the concept of ethnomedicine by using traditional plant species as medicinal natural sources. As a result, almost half of the products in the drug market today are derived from bioactive natural compounds. In addition, natural biomolecules are widely used as lead compounds for the synthesis of new bio-inspired active compounds, which could be an ideal alternative to synthetic drugs in terms of cost and efficacy [1-3].

According to projections, an increasing trend in the number of novel natural chemical substances is predicted due to the breakthroughs in *in silico* screening and drug discovery field [1-3]. Some of the most prominent examples of plant-derived medicinal products are antimalarial drugs based on artemisinin and quinine analogues, antihypertensive compound reserpine and anti-asthma derivatives of ephedrine. Furthermore, natural components with anticancer properties like alkaloids vinblastine and vincristine have already found clinical uses. Other natural agents with chemoprotective activity originated from plant and marine organisms such as topotecan, ecteinascidin 743, halichondrin B, have been used as starting points for the discovery of novel anticancer drugs,

being already in the stage of clinical trials [1]. Up to 2014, Food and Drug Administration (FDA) 45% of drugs against cancer where either natural products or derivatives of natural products [4] (Fig. 1.1).

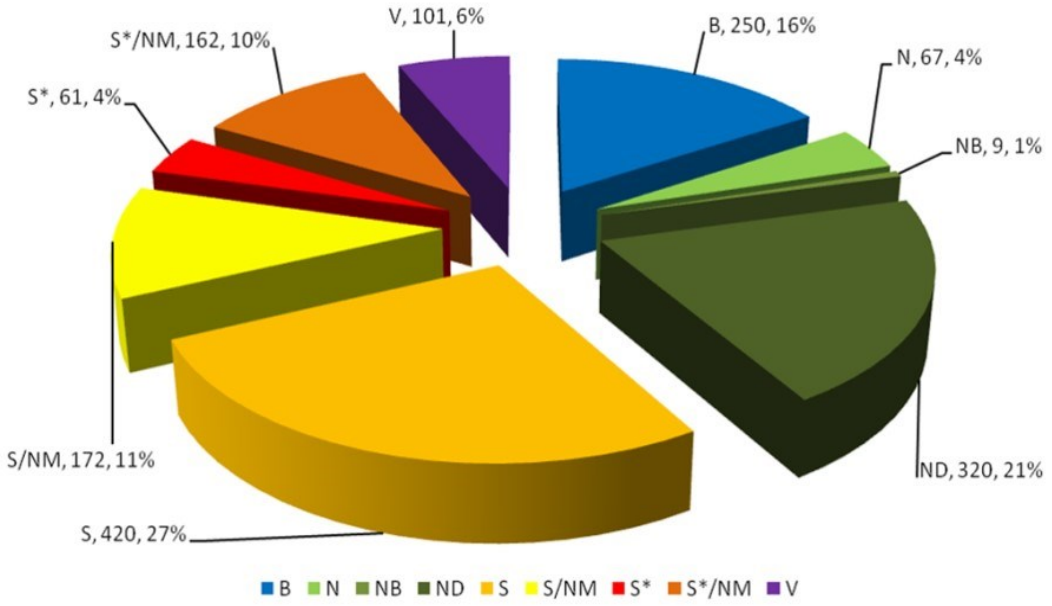



**Figure 1.1. All approved drugs from 1981 to 2014, where B: Biological macromolecule, N: Natural product, NB: Botanical drug, ND: Natural product derivative, S: Synthetic drug, S*: Synthetic drugs based on natural product pharmacophore, V: Vaccine, NM: Mimic of natural product [4]**

Among all related industries, food, nutraceuticals and cosmeceutical companies hold the lion's share of natural products' market according to forecasts that predict an over 10% growth until 2026 [4]. However, the intensive use of plant- or animal-derived natural ingredients pose ecological and social considerations since the population of cultivator or producer countries faces, in many cases, the threat of wild crafting, unsustainable harvesting practices, undernourishment and increased food prices [5]. At the same time, European Union reports revealed that almost 70% of total food processing ends up as waste or byproducts and foresaw an increase of 30% at the disposal of food byproducts by 2020 [6]. Thus, the exploitation of natural byproducts emerge as an ideal solution in terms of sustainability and economic impact. In addition, the aforementioned global challenges forged the concepts of 'food from food' and 'byproducts to co-products' through the low-waste industry production and the establishment of sustainable management strategies that are focused on the eco-procurement, supply chain management, waste minimization and resource

efficiency actions by implementing modern methodologies for the extraction and valorization of therapeutic natural agent from natural byproducts [7-9].

## 1.2 Examples of food products of high bioactive content and high-added value natural byproducts

Over the last years, different epidemiological studies highlighted the significant role of a nutrition rich in foods with high content of bioactive ingredients. The dietary intake of certain classes of bio-nutrients by incorporating various groups of food in a daily diet is highly recommended due to their potential beneficial properties against several types of cancer, cardiovascular diseases, diabetes, hypertension, eye impairments and inflammations [10]. Herbs (tea, sage, rosemary, cinnamon, thyme, etc.) [11], fruits (apples, grapes, pomegranate, apricots, berries, tropical fruits, etc.) [12], vegetables (tomatoes, olives, broccoli, onions, spinach, carrots, peppers, etc.) [13], cereals (wheat, rice, corn, etc.) [14], seafoods (shrimps, lobsters, crabs, oysters, salmon, etc.) [15], mushrooms [16] and lipid foods (avocados, eggs, vegetable oils, nuts, etc.) [17] are the major food classes which contain a plethora of biofunctional compounds (i.e. vitamins, phenolics, carotenoids, phytosterols, polysaccharides, fatty acids, saponins).

The processing of these food categories generates an enormous amount of wastes and byproducts, sometimes of even higher bioactive content compared to the edible products. Therefore, the commercialization of agro-, animal- and seafood residues offers many opportunities regarding the enhancement of the local economy and the increase of the surplus revenue of the pertinent companies [6].

Citrus peel is one of the most distinctive natural byproducts used for the recovery of essential oils, pectin, sugars and polyphenols. Tomato and tomato-based industry produces significant quantities of tomato pomace, which is mainly consists of skin, seed and pulp rich in carotenoid lycopene, known for its immunomodulatory, anti-inflammatory and antioxidant activity [18]. The high antioxidant and antimicrobial content of pomegranate renders it as a significant agro-industry product. Pomegranate arils, peel and seeds contain a variety of different bioactive compounds, such as phenolic acids, flavonoids,

anthocyanins and fatty constituents of seed oil, which present antibacterial, antiatherosclerotic, inhibitory, antioxidant and skin-protective properties [19].

Three to four million cubic meters of olive-oil production waste (i.e. olive pruning residues, olive mill wastes, olive pomace, olive leaves) are generated annually in the Mediterranean area and are characterized by high organic load, toxicity and recalcitrance. Nonetheless, olive-oil byproducts are rich in flavones, alkaloids, secoiridoids, small phenolic alcohols, sterols, tocopherols, squalene, lignans and phospholipids, compounds which are designated as natural health-promoters [20]. Additionally, several hundred thousand tons of grape pomace or marc, which is the main solid by-product deriving from winemaking, are produced in Greece annually within a three-month period and is usually accumulated as waste together with grape stalks [21]. Winery residues, from both white and red grape varieties, are substrates containing hydroxybenzoic and hydroxycinnamic acids, anthocyanins, flavan-3-ols, flavonols and their glucosides/glucuronides/rhamnosides/rutinosides, tannins and their dimmers or trimmers [22]. These compounds act mainly as anti-inflammatory agents by reducing secretion of inflammatory mediators (INF-γ, IL-6), suppressing the expression of IL-1α, TNF-α, IL-8 pro-inflammatory cytokines, promoting the inhibition of leukocyte migration and enhancing the anti-inflammatory cytokine IL-10 production [23].

Crustaceans industry create an important volume of under-utilized byproducts (cephalothorax, carapace, tail, shells, etc.), which are comprised of economically important biomolecules, like carotenoids, proteins and polysaccharides (chitin, chitosan) used as films for active packaging, metal chelators and antimicrobial agents [24].

Besides their utilization in the nutraceutical and cosmetics sector, natural substrates could be also used in other industrial applications for producing enzymes and biofuels, nanoparticles, biodegradable plastics, natural coloring and flavoring supplements, food formulations and animal feed [6].

In the current project, the natural substrates under study include 1) agro- and seafood byproducts (apricot pulp and shrimp head) and 2) lipid foods (egg yolks and shrimp body).

### 1.2.1 Apricot and apricot byproducts

Apricots (*Prunus armeniaca*), a fruit widely cultivated in the Mediterranean and Asian countries, is one of the primary representatives of the fruit market with numerous products including raw fruits, jams, spreads, canned fruits, juices, fresh preparations, etc. Apricots are a climatic fruit with a relatively short harvest period of maximum two months during early summer. Although there are many different varieties, their cultivation and production are not very adaptive in different climatic conditions since they are usually grown under severe long winters and dry summers or at short mild winters and hot dry summers. In addition, apricots cultivars are really susceptible to various diseases (European dry rot, bacterial spot, plum pox, fungal and bacterial canker, shothole, etc.) [25]. Thus, the crosses between different apricots varieties and the advances in genetic engineering should be used for the introduction of new cultivars more resistant in diseases and with enhanced bioactive content.

Despite the fact that apricots' therapeutic effect has not yet be proven, there are preclinical and animal studies, which indicate inhibition of cancer cells, decrease of the liver injury in hepatic steatosis, anti-inflammatory effect against inflammatory bowel diseases, antimicrobial and antibacterial activity [26].

Apricot byproducts are a total of non-edible parts, fruits of non-acceptable sensory characteristics and fruits which are over- or under-ripe [27]. They are mainly constituted from pulp, peel and apricot kernels. The oil of apricots kernel is used in food and pastry industry due to its high content in amygdalin, a characteristic compounds responsible for almonds unique taste [28].

Reports by Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) place Greece at the top twenty producer and exporting countries of apricots worldwide. The variety 'Bebekos' holds the 70% of Greek production followed by the varieties 'Tyrinthos' and 'Luizet'. As a sequent, large production size generate immense quantities of apricots byproducts with high contents mainly of carotenoids and sugars, then secondarily of phenolics (rutin, catechin, epicatechin and chlorogenic acid) [28] and amino acids. Focusing on apricots pulp carotenoids, the orange color unveil the presence of carotenes (a- b- and γ- carotene, etc.) rather than xanthophylls (zeaxanthin, lutein, b-cryptoxanthin,

etc.). Among a- and b-carotene, the b-isomer is the prevailing form in plant tissues [18]. According to published works, xanthophyll zeaxanthin is the second most abundant carotenoid in apricots, but its content can be even 50-times lower than that of b-carotene [29]. Other apricot carotenoids present in amounts <2% are phytoene, phytofluene, *γ*-carotene, lycopene, a-cryptoxanthin and lutein [30]. Thus apricots byproducts may be accede to a low-cost, easy to find and sustainable natural source of potential bioactive compounds [18].

### 1.2.2 Shrimp and shrimp byproducts

Shrimps are invertebrates, whose carapace and body contain high amounts of various bioactive compounds, principally carotenoids, fatty acids, proteins and peptides, of significant nutritional value. Asian countries like Thailand, India and China farm, process and export tons of shrimps with a revenue of billions annually [31].

Shrimp body is considered an invaluable source of proteins, carotenoids, minerals, cholesterol and ω-3 essential unsaturated fatty acids (eicosapentaenoic and docosahexaenoic acids) and therefore is acknowledged as a high-value aquaculture product. Normally, shrimp body is separated in processing plants from shrimp head for exportation purposes disposing this way around 40-50% of the total weight of raw material [32].

The accelerated growth of crustaceans' food industry and the imminent waste production provoke an important ecological problem in these regions by threating certain shrimp species with extinction and by evoking the fast degradation of head protein content to biogenic amines [32].

Both shrimp head and body are important natural sources of carotenoids, the main of which are astaxanthin, canthaxanthin and their esterified forms. Especially, astaxanthin has 10 times and almost 500 times stronger antioxidant activity than b-carotene and vitamin E [33].

Hence, the recovery of bioactive components from shrimps and their byproducts can form a sustainable financial and environmentally friendly alternative for the isolation of these compounds and their use especially as natural food additives and animal feed [34].

### 1.2.3 Egg yolk

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 [35]. 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 [36].

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 [37, 38]. Egg yolk is a lipid food rich in carotenoids lutein and zeaxanthin, known for their activity against visual impairments. Therefore, their dietary intake through carotenoid-rich foods and supplements is strongly recommended as preventive approach [39].

Egg yolk of a medium size egg weighs around 20 g and contains 200-400 μg of these carotenoids [40]. The variances in their content depend on numerous factors, mainly on hen's diet. Other parameters affecting carotenoids content are egg weight, feeding systems, type of breeding (traditional commercial and organic eggs), conditions of housing and management systems, avian species, etc. In European countries, poultry feed can be enriched with either synthetic zeaxanthin or natural zeaxanthin, received from corn. In addition, canthaxanthin, another feed additive carotenoid, could be detected in egg yolk. Canthaxanthin is an approved synthetic additive for the enhancement of egg yolk color, permitted at concentrations ≤66 mg/kg of solid or semisolid food and ≤4.41 mg/kg for broiler chicken [41].

Due to its unique health-promoting functions and its high lipidic content, egg yolk emerges as the most adequate lipid substrate for reviewing carotenoids delivery, absorbance and interaction with lipid constituents in humans.

## 1.3 Bioactive compounds of natural products

By the term "bioactive compounds," we refer mostly to secondary and some primary metabolites, which exhibit certain biological effects and act as

functional ingredients in low concentrations. A generalized classification, based on chemical structures, would assort them to a number of different groups, like:

i) phenolic acids and polyphenols (tannins, anthocyanins, flavonoids, stilbenes etc.)

ii) carotenoids

iii) terpenes, essential oils and terpenoids,

iv) fatty acids and lipids,

v) amino acids and proteins,

vi) poly-, di- and monosaccharides

vii) alkaloids

viii) lignans and sterols [2].

Nature is an inexhaustible source of these molecules, which are present in different parts of vegetables, fruits, flowers, plants and herbs, animal products, wine and winery by-products, marine organisms, algae, bacteria, crustaceans, eggs, and plant oils [42, 43]. Furthermore, since the amount of waste produced by agro-industry and seafood sectors reaches excessive numbers, natural byproducts constitute a really cheap source of biomolecules and also a sustainable, profitable, and eco-compatible idea for creating health-beneficial co-products from residuals of natural origin [44, 45].

But why are these molecules so important? Modern drug research is based on bioactive compounds from natural products since these components are known, among other, for their therapeutic effects including antimicrobial, antihypertensive, anticancer, cardioprotective, antioxidant, antidiabetic, neuroprotective, chemoprotective, antiaging, and immunoregulatory activity [42, 46]. They can also act as functional food constituents, coloring, flavoring, and preserving food additives, fragrances, authenticity indices, active packaging agents and biomarkers in metabolic pathways [47]. Recent studies show that their high bioavailability enhance their potential health benefits and this is the reason why countries that follow Mediterranean diet rich in bioactive ingredients, present a lower percentage of morbidity and mortality caused by cancer, cardiovascular and neurodegenerative disease [48].

In more details, phenolic acids (i.e. caffeic acid, ferulic acid, chlorogenic acid, rosmarinic acid) present significant antioxidant effect. Clinical trials confirm the inhibitory activity of flavonoids against cancer proliferation. Flavones (luteolin, apigenin, etc.) and flavonols (quercetin, myricetin, kaempferol, etc.) reduce the risk of strokes and myocardial infarction. Anthocyanins are recognized as natural immunomodulators through the reduction of inflammation. They also protect DNA from undesirable modifications and reduce estrogen activity. Stilbenes, like resveratrol, have proven antiaging properties [49].

Triterpenoids (oleanolic acid, ursolic acid, betulinic acid, etc.) target various androgen-related diseases, like prostate cancer, benign prostatic hyperplasia, acne and hirsutism. Terpenes (limonene, pinene, cymene), which are the main hydrocarbons of essential oils, are the principal components of perfumes and insect repellents. They also promote normal cell growth and are antifungal, antibacterial, spasmolytic and antiviral agents [50]. Alkaloids (i.e. atropine, morphine, quinine, vincristine) exhibit a wide range of biological functions. Among them, the most prominent are their anti-proliferative activity and their positive effects against hypertension, postpartum bleeding, sensile cerebral insufficiency and other clinical incidents. The consumption of phytosterols (b-sitosterol, campesterol, and stigmasterol) reduces cholesterol levels, while clinical trials indicate their immunological properties against breast and colorectal cancer. Lignans (lariciresinol, matairesinol, pinoresinol, syringaresinol, etc.) are known for their antiviral, antitumor, antimicrobial and antimitotic effects [49].

Proteins and peptides are used as natural components for the improvement of the nutritional value of conventional food products and the manufacturing of products with tailored functionalities (milk, cheese, infant foods). Recently, whey proteins have been used in nanocarriers systems in pharma- and nutraceutical applications. In addition, iron-containing protein lactoferrin present numerous bioactivities (antibacterial, antiparasitic, antifungal, anti-inflammatory activity) [51].

Polysaccharides (β-glucans, starch, cellulose, chitin, chitosan) demonstrate significant pharmacological properties such as antitumor, hypoglycemic, anti-fatigue, anti-complement, anticoagulation activity and stimulation of probiotic

bacteria growth. Fatty acids, oils or other lipid classes find many applications in cosmetic and personal care products (cream, shampoos, conditioners, soaps) as they have moisturizing and skin-protective properties. They are also related to the treatment of arthritis, hypertension and coronary diseases [52].

Carotenoids are the bioactive compounds of interest in the present research, therefore their chemistry, role and biological activities are described thoroughly in Chapter 2.

# CHAPTER 2
# CAROTENOIDS: CHEMISTRY, OCCURRENCE AND BIOLOGICAL ACTIVITIES

## 2.1 Carotenoids: General information

Carotenoids are $C_{40}$ symmetrical molecules of tetraterpene group, which consist of eight isoprenoid units and an extended conjugated double bond system with delocalized π-electrons that act as chromophore [53]. As natural lipophilic pigments, carotenoids are responsible for the red, orange and yellow coloration of fungi, higher plants, herbal and animal natural products, like fruits, vegetables, egg yolk, marine organisms, insects, birds and fishes [54]. However, in higher marine or animal organisms, carotenoids cannot be synthesized *de novo*, but they can be accumulated through metabolic transformations by the consumption of organisms that can produce carotenoid, for example algae and bacteria or various plants. According to clinical studies, humans can absorb and metabolize more than 40 different carotenoids [55], which are detected in serum, milk and tissues. For example, human serum contains around 20% of lutein and lycopene, 10% of b-carotene, 8% of b-cryptoxanthin, 6% of a-carotene and 3% of zeaxanthin [56].

Namely, carotenoids participate at the photosynthetic process of fungi, bacteria, algae, yeast, molds and plant occurring in their chromoplasts and chloroplasts. Specifically in plants, leaf color is attributed to carotenoids after chlorophyll degradation. Furthermore, during fruits ripening, the free carotenoids forms are converted to esterified carotenoids-fatty acids molecules. Up to present, more than 600 structures of carotenoids have been identified, but still there is an ongoing research for the discovery of new carotenoids compounds [55].

Based on their chemical structure, they are classified into (a) **hydrocarbon** carotenoids (lycopene, carotenes) and (b) **oxycarotenoids**, which also contain oxygen in their structure, known as xanthophylls [57]. The latter class includes subgroups according to the functional group added in the carbon chain. The most common functional groups are (i) hydroxyl-group (cryptoxanthin, lutein, zeaxanthin) (ii) keto-group (canthaxanthin) (iii) combination of hydroxyl- and

keto-group (astaxanthin, capsanthin) (iv) epoxy-group (violaxanthin, flavoxanthin) (v) alcohol ester-groups (fucoxanthin, astacein) (vi) carboxyl-group (bixin, crocetin). The last subgroup is included in an additional carotenoid group, known as apocarotenoids [6]. Another classification of carotenoids is based on their activity as precursors of vitamin A. Carotenoids containing non-substituted β-ionone rings (a-b-carotene, cryptoxanthin) can be converted to retinal, while carotenoids lacking these rings are known as non-provitamin A carotenoids (lutein, zeaxanthin, lycopene, astaxanthin) [6]. The predominant form of these compounds in nature is the *trans*-configuration, but also *cis*-configuration of different biological properties exists, when *trans/cis*-isomerization takes place due to heat or light [58].

Apart from functioning as natural pigments, carotenoids are considered bioactive compounds, as they exhibit an antioxidant and antiaging activity, play a preventive role against tissue damages and chronic diseases, especially in eye [56] and cardiovascular diseases [59, 60], diabetes [61, 62] and certain types of cancer [63, 64]. They also protect foods from degradation and stabilize them. Moreover, they can be used as constituents in functional food products [6].

## 2.2 Carotenoids biosynthesis, degradation and bioavailability

Carotenoids biosynthesis involves different reaction steps and pathways including the plastid-specific 1-deoxyxylulose 5-phosphate pathway, phytoene and lycopene biosynthesis pathways, desaturation, cyclization and hydroxylation reactions [55]. The mechanism of carotenoids biosynthesis is presented in Figure 2.1.

The long polyene blocks in carotenoids chain are responsible for their relatively easy degradation, which depends on many factors. Heat, light and acids are key factors to geometrical isomerization (*trans*- to *cis*- isomerization) of carotenoids. Oxygen promotes carotenoids oxidation through epoxidation reactions and degradation to apocarotenoids, which subsequently form low molecular weight compounds liable for the unsavory flavor of fruits and vegetables. Moreover, carotenoids cleavage occurs by different enzymatic reactions, mainly dioxygenases oxidation [55]. The products formed from

carotenoids degradation are also shown in Figure 2.1.

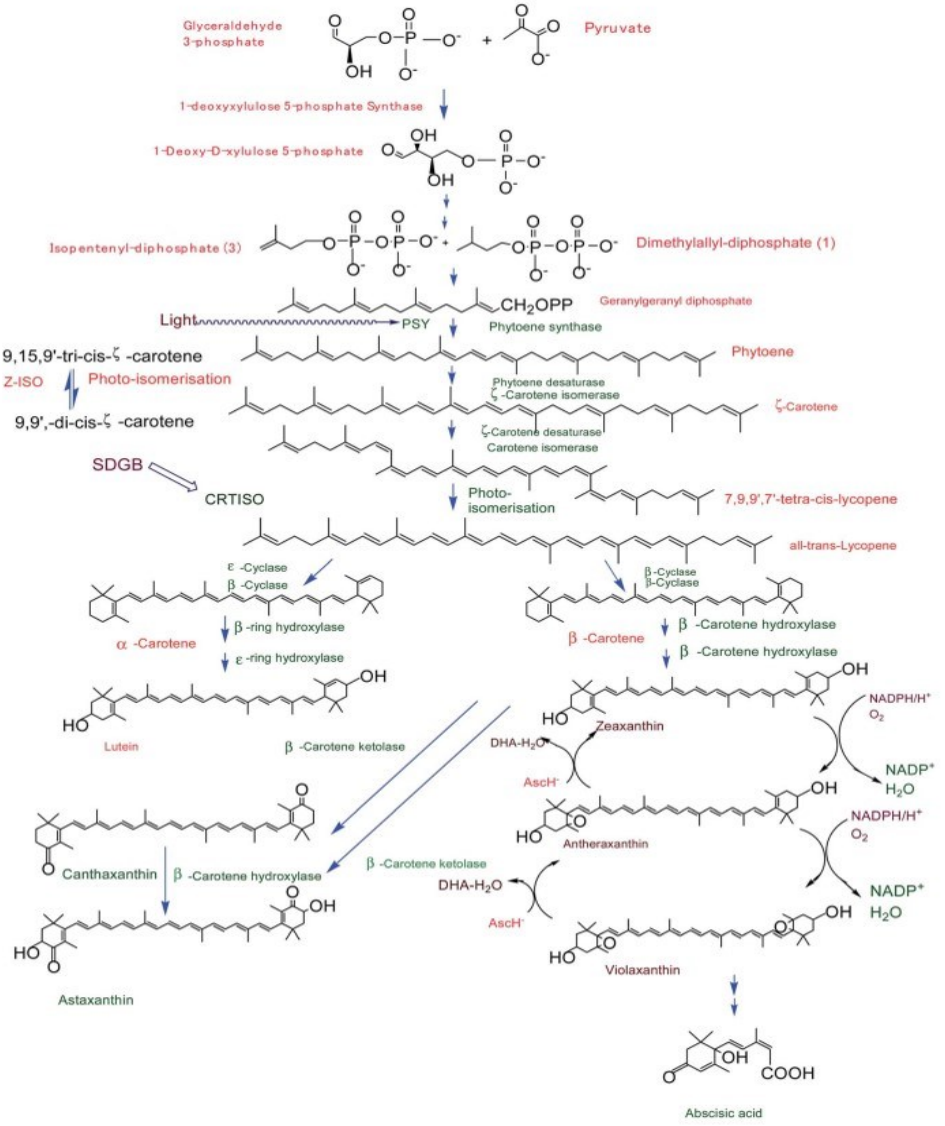



**Figure 2.1 Mechanisms and products of carotenoids biosynthesis and degradation [55].**

Bioavailability refers to the moiety of an ingested nutrient that is used by the body in order to follow through a series of physiological actions. Although carotenoids' bioavailability is strongly related to various parameters (carotenoid structure, matrix nature, molecular linkage, quantity of carotenoids contained in a meal, factors affecting the absorption and bioconversion of carotenoids, genetic factors of individual, etc.), the most crucial parameter is the type of diet since carotenoids are fat-soluble molecules. Thus their absorption and bioavailability is higher when lipid foods are consumed instead of plant tissues. *cis*-Isomers are less absorbed than the corresponding *trans*-forms due to *trans*-

isomers increased solubility in bile acid micelles. In addition, evidences indicate the antagonistic absorption of carotenoids when more than one is received. For example, canthaxanthin hindered lycopene's uptake. Hence, the different rate of carotenoid absorption should be ascribed to deposition mechanisms, distinct for each carotenoid [55].

## 2.3 Carotenoids of substrates under study

This project is focused on the study of the major carotenoids contained in apricot byproducts, shrimps and egg yolk. The properties and biological activities of these carotenoids is described in sections 2.3.1-2.3.5. Their chemical structures are illustrated in Figure 2.2.

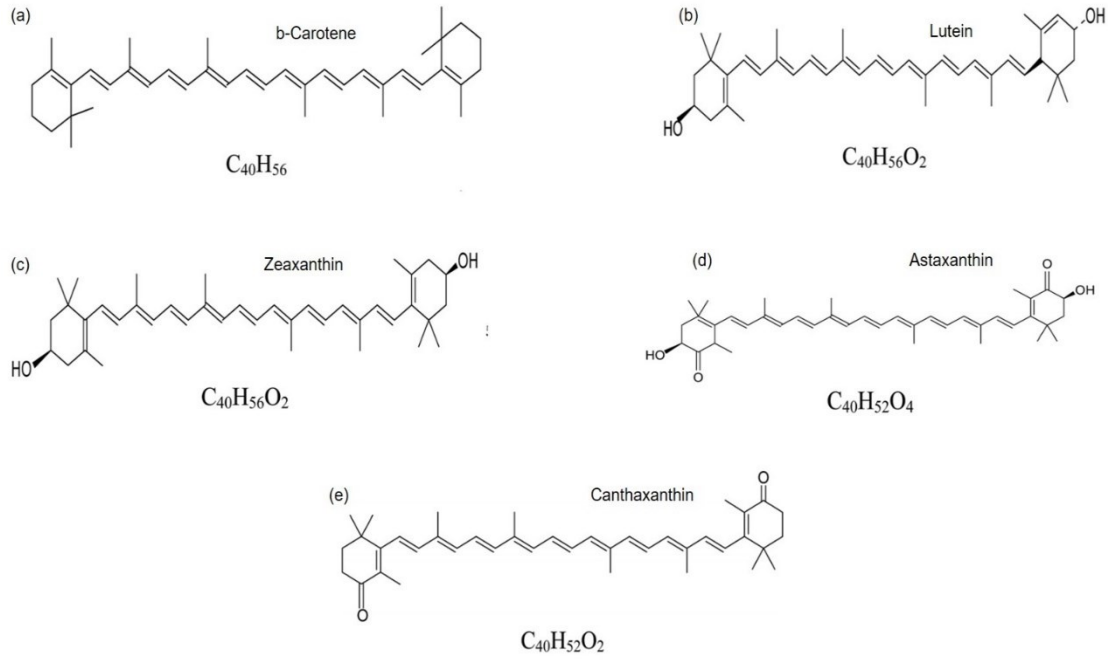



**Figure 2.2. Carotenoids chemical structures [55].**

### 2.3.1 Beta-Carotene (b-carotene)

Beta-Carotene (b-carotene) (Fig. 2.1a) along with lycopene and phytoene are the main hydrocarbon carotenoids. b-Carotene has the double bonds of the two β-ionone rings in different locations compared to a-carotene and ε-carotene. Gamma-carotene (γ-carotene) and δ-carotene lack one of the two ionone rings of b-carotene [65].

Beta-carotene acts as antioxidant and major precursor of vitamin A (retinol), since it presents 100% provitamin A activity due to its two β-ionone rings [18].

It enhances the immune system by regulating intercellular signaling pathways, cell differentiation, growth factors and cell apoptosis. Also it offers protection against atherosclerosis, coronary diseases, toxin formation and accumulation in the liver [6].

In addition, there are evidences for the significance of b-carotene metabolic pathways in the risk reduction for some types of cancer (lung, head, prostate, skin, liver, breast, colorectum). Nonetheless, in order to avoid negative effects, many clinical intervention studies, recommend low-dosage dietary uptake levels of b-carotene supplements [64]. Cases of multi-carotenoids supplementation indicated positive results on Alzheimer diseases and vision impairments by balancing the adverse effects of different kinds of degeneration, UV radiation, malfunctions and oxidative stress [18]. Concerning visual impairments, like age related macular degeneration, the recommended b-carotene intake is between 15-60 mg per day [55].

Green leafy vegetables and orange fruits and vegetables are an abundant source of b-carotene. Some typical examples of plant tissues of important b-carotene content are apricots, carrots, citrus, spinach, pumpkins, sweet potatoes, paprika, chilli and so on [55].

### 2.3.2 Lutein and zeaxanthin

Lutein (β,ε-Carotene-3,3'-diol) (Fig. 2.1b) and zeaxanthin (β,β-carotene-3,3'-diol) (Fig. 2.1c) are xanthophylls' isomers, whose difference is assigned to the position of the unsaturation of the double bond at their end ring. Lutein consists of three chiral centers, which allows eight different stereoisomeric configurations. Nonetheless, only Z-*cis*-(R,R,R) form is present in nature. On the contrary, zeaxanthin exists only in three different stereoisomers, namely (R,R)-, (S,S)- and (R,S)-*meso*-zeaxanthin, despite of its two chiral centers. Besides the different location of the unsaturated double bond, lutein comprise of one β-ionone ring and one ε-ionone ring, while zeaxanthin contains two β-ionone rings [56].

The health-promoting effects of lutein and zeaxanthin, have been well established mainly due to their antiiflammatory [66] and antioxidant properties which enables their chemoprotective action and beneficial activity against

cardiovascular and Alzheimer's diseases [64]. Furthermore, these carotenoid isomers are important bioactive ingredients in novel cosmeceutical preparations with confirmed positive effects on skin hydration and elasticity [67]. Recent studies revealed also a relation between macular xanthophylls and increased bone mass in young adults [68].

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), diabetic retinopathies, glaucoma and cataract) worldwide will be doubled by 2030 [40]. 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 [39]. Conforming to nutritional and medical surveys (i.e. National Health and Nutrition Examination Survey) an individual 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 and zeaxanthin amount of almost only 2 mg [69].

Normally lutein content in dietary sources is five times higher than zeaxanthin. In foods containing fats and lipids (egg yolk), xanthophylls and their esters are present in lower yields, but they show higher bioavailability compared to foods of plant origin (e.g. spinach, broccoli, corn and kale) [35]. 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 [70]. According to scientific findings, lutein eye concentration reaches an increase of over 20% in cases where egg consumption is daily for a 3 months period [56]. Thus, current drug formulations contain lutein encapsulated in solid lipid nanocarriers, phospholipid suspensions and nanostructure lipid carriers [37, 38].

### 2.3.3 Astaxanthin

Astaxanthin (β-Carotene-4,4'-dione) (Fig. 2.1d) is a xanthophyll containing both a hydroxyl- and a keto-group. The presence of two chiral centers permits the formation of two enantiomer configurations (3R, 3′R, and 3S, 3′S) and one *meso*-form (3R, 3′S) present in a racemic mixture [6].

Astaxanthin is the major subunit of carotenoproteins, which are macromolecules composed by the imine bond between astaxanthin and proteins. Beta-crustacyanin (β-crustacyanin), and a-crustacyanin, present in lobsters and other crustaceans, are two carotenoproteins fully elucidated. The first carotenoprotein is an astaxanthin-apoprotein dimer, while the second complex is an octamer of β-crustacyanin. Carotenoproteins could be identified simply by visible spectrophotometry due to the high bathochromic effect caused by the bound protein, that results in different absorbance wavelengths (580-630 nm) compared to the free carotenoid molecule (440-490 nm) [6].

Astaxanthin exhibits similar biological activities to the other carotenoids besides that of provitamin A. Antioxidant, anti-inflammatory and anti-degenerative effects associated with cardiovascular disease, arterial stiffening, atherosclerosis and Alzheimer disease are numbered among its beneficial effects [60]. In addition, astaxanthin present gastro- and hepatoprotective effect, antidiabetic activity against diabetic nephropathy and chemopreventive properties against colon, breast, prostate, embryonic fibroblasts and oral fibrosarcoma cells [62]. It should also be considered as a vital component in various cosmeceutical products (hand creams, shampoos, sunscreens, sun gels, toothpastes, lotions) since it is recommended as an antimicrobial, anti-wrinkle, antiaging, anti-acne, moisturizing, sun-protective, anti-pigmentation agent [71].

Marine bacteria, red yeast, microalgae (*Haematococcus pluvialis*) are natural producers of astaxanthin, which is then ingested by fishes and seafood, explaining this way its occurrence to krills, salmon, lobsters, mussels, shrimps, crabs and avian species, like flamingos. Especially in crustaceans, astaxanthin is present mainly in mono- and di-esterified forms with various fatty acids, such as lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), oleic

acid (C18:1, ω-9), arachidic (C20:0), eicosapentaenoic (C20:5, ω-3) and docosahexaenoic acids (C22:6, ω-3) [6].

### 2.3.4 Canthaxanthin

Canthaxanthin (β-β′-carotene-4,4′-dione) (Fig. 2.1e) is a diketo-xanthophyll with nine conjugated double bonds, which absorb the light and provide the red-orange color of this compound. In nature, canthaxanthin appears in *trans*-form and in *cis*-geometrical isomers (9-Z-, 9,9′-di-Z-isomers). Oxidation of canthaxanthin produce 4-oxo-β-apo-carotenals, 4-oxo-β-apo-carotenones and 5,6-epoxy compounds [72].

The presence of two keto-groups increase the polarity of canthaxanthin and enables its distribution to tissues, like liver and skin, via low- and high density lipoproteins. The more polar structure of this molecule is responsible for its biological functions. Canthaxanthin proved to have free radical scavenging, and anti-atherogenic ability by modulating the cholesterol-induced oxidation. It also plays an important role in immune system against DNA damage, infectious diseases and chronic inflammation. Canthaxanthin promote the gap junction formation between cells and thus it presents gastro-protective, anticancer and cell signaling functions [72].

Along with astaxanthin, canthaxanthin is used as a feed additive to improve fish and eggs' color in quantities regulated by consumers' safety guidelines of European Union. According to the evaluation of the European Food Safety Authority (EFSA), the dietary use of canthaxanthin is permitted at concentrations from 5 to 30 mg/kg depending on the type of food. However, consumers should avoid canthaxanthin-containing tanning pills since they are related to ocular damages [72].

Like astaxanthin, canthaxanthin is also produced by microalgae and is present to different seafood species (crustaceans, fishes, etc.) [55].

# CHAPTER 3
# HIGH ENERGY EXTRACTIONS-EXPERIMENTAL DESIGN

## 3.1 Extraction: The cornerstone for recovery of bioactive compounds

Extraction process is the key point for successful recovery of bioactive compounds since ancient times. Its fundamental principal is based on the targeted retrieval of certain chemical groups from a solid or liquid matrix by a liquid solvent. Extraction efficiency is strongly related to variables, like extraction duration, pressure, temperature, solvent system and substrate nature. Extraction techniques are of utmost significance in the workflow of a validated analytical technique as they are characterized as “sample preparation” methods. Research departments in pharmaceutical and cosmetic companies use extraction processes as the first step for the targeted isolation of compounds right after *in silico* studies and before biological activity tests that will allow the promotion of new formulations in the market [2] (Fig. 3.1).

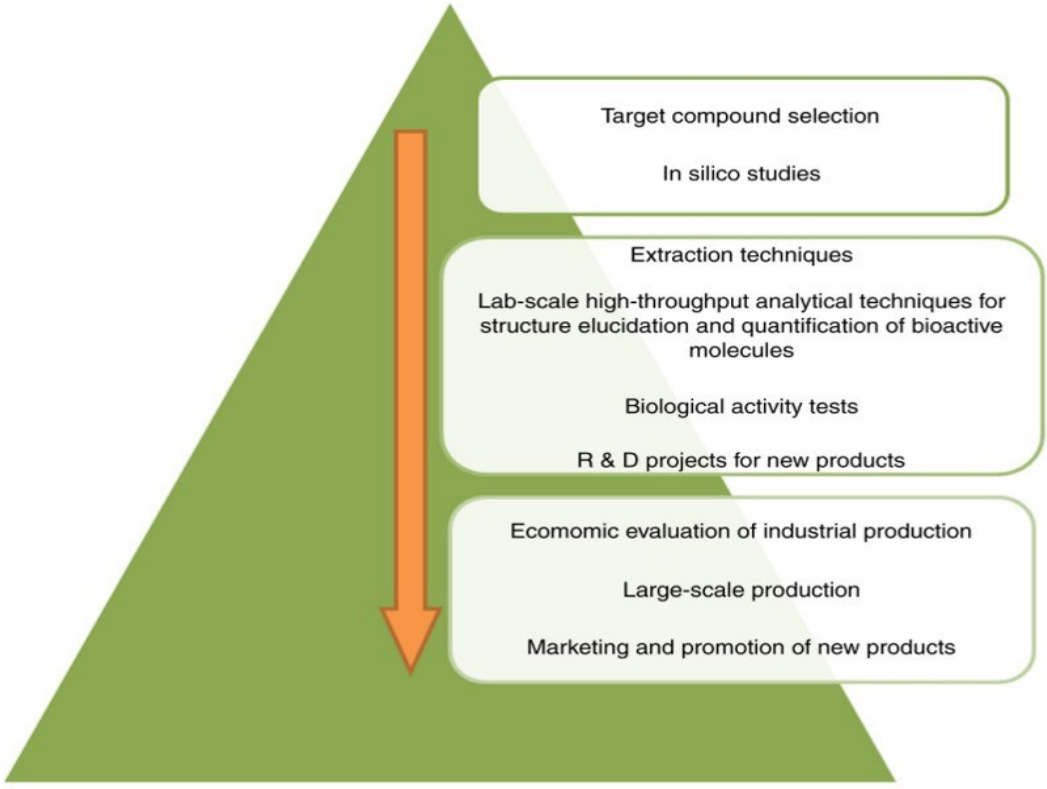



**Figure 3.1. Bioactive compounds from natural products: From lab-research to the market [73].**

A number of various extraction methods, from traditional procedures to contemporary nonconventional techniques, are recorded and used in modern chemistry applications. Classic extraction methods include classical diffusion through steering, maceration, distillation, and specific modifications of Soxhlet, Bligh-Dyer and Folch procedures, while modern approaches enclose supercritical (SFE) and accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), subcritical water extraction (SWE) and pulsed electric field extraction (PEFE). Also liquid microextraction techniques, like solid phase microextraction (SPME), stir bar sorptive extraction (SBSE), liquid phase microextraction (LPME), dispersive liquid–liquid microextraction (DLLME), extrusion, membrane-assisted extraction (hollow fiber renewal liquid membranes, HFRLMs/hollow fiber strip dispersion, HFSD), and enzymatic extraction belong to this group of extraction practices [44].

The majority of these techniques refer to high energy extraction, a fast and nonconventional way to give high amounts of energy to a chemical system. Some of these techniques, like SFE and PLE, require heating of the extraction mix in order to isolate target-molecules, while others, like UAE and MAE, are described as non-thermal extraction methods. However, all these methods belong to what is called “green extraction practices,” which are defined according to Chemat et al. (2012) [44] as “extraction practices based on the discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product.” Modern extraction techniques tend to sideline traditional methods as they are more selective, ecofriendly, fast, energy-saving processes with less solvent consumption and better extraction yields. As green practices, high-energy techniques comply also with other requirements of green chemistry, like instrumentation and industry extraction units’ diminution, automatization and monitoring of extraction processes, solvent recycling, exploitation of waste and residues by introducing innovative coproducts, and application of pioneering technologies. Nonetheless, contemporary research should focus on improving repeatability, accuracy, and reproducibility of modern techniques because

classic extraction methods are still acknowledged and used in large-scale units as robust and reliable reference methods [2, 44, 74-76].

## 3.2 High energy extraction: A modern approach for recovering bioactive compounds

The 21st century inaugurated a new era for drug and food companies with the introduction of products based on molecular structures of natural origin known for their reported functional and therapeutic effects. The group of nutraceuticals includes natural nonfood pharmaceutical formulations, like dietary supplements that contain high concentrations of bioactive molecules present initially in food substrates. Due to high amounts of bioactive compounds contained in these preparations, their health-promoting outcome is more intense compared to that of their original source. Additionally, they present minimum detrimental side effects when set side-by-side with similar synthetic formulations. Furthermore, foods or components of everyday diet, which demonstrate beneficial health effects besides their elevated nutritional value, are characterized as functional foods. Their advantageous properties are drastically correlated to bioactive compounds presence [77].

High energy extraction methods are the building blocks of nutraceuticals and functional foods production since the amount of energy delivered to the system could cause matrix cell rupture and more quantitative release of the natural compounds. These techniques came as an environmentally friendly and sustainable remedy to the drawbacks of the old-fashioned techniques [2].

Soxhlet extraction, maceration, hydrodistillation, and their modifications are used for more than a century as classic extraction approaches for the recovery of bioactive ingredients. All these methods rest on the molecular affinity between target compound and used solvent, therefore adequate solvent selection is of utmost importance. They are simple and inexpensive practices with no need of filtration, which results in quite high yields due to the continuous fresh solvent addition and the achieved high temperatures. Nevertheless, conventional extraction techniques are (i) laborious since extraction process cannot be accelerated, (ii) environmentally unsafe, (iii) not fully automated, (iv) solvent-consuming methods that demand (a) large amounts of expensive

organic solvents and (b) extra operational units for solvent removal. Additionally, they do not always provide protection to thermolabile compounds (i.e carotenoids) and they do not present high selectivity toward specific compound groups, since along with active molecules, several impurities could be extracted. [2, 78, 79].

| Levels of Extraction Influential Factors | Conventional Techniques | Nonconventional Techniques |
|---|---|---|
| Extraction efficiency | High | High |
| Extraction time | Hours to days | μs to less than 1 h |
| Extraction parameters | Time, solvent system, solvent/material ratio, temperature, particle size, pH | Time, solvent system, solvent/material ratio, solvent flow, temperature, pressure, particle size, pH, energy power, extraction mode, sample moisture |
| Thermolabile compound protection | Low | High |
| Sample size | Medium to high | Medium to small |
| Operational cost | Low | Medium to high |
| Operational difficulty level | Low | Medium to high |
| Automatization | Low | High |
| Need for additional treatment steps | High | Low |
| Environmental impact | Energy- and solvent-intensive, negative environmental effect | Energy- and solvent-saving, ecofriendly |

**Figure 3.2. Comparison of conventional and nonconventional techniques [73].**

On the other hand, modern extraction approaches are focused on (a) achieving increased extraction yields, (b) shorter extraction times, (c) greener extraction solvents, (d) minimized energy and solvent volumes, (e) wide range of extractable analytes, (f) improved selectivity, (g) cost-saving non-elaborated processes, (h) thermoprotection of sensitive compounds and (i) downscaling of operational units in industrial processes through new set-ups and technologies [8, 80]. A comparative study of conventional and modern techniques is presented in Figure 3.2.

As high energy extraction fulfills green chemistry concepts and practices, these techniques are emerging as the most suitable way for eliminating any traditional polluting extraction technology and for off-line waste management in industrial level [81]. The new extraction technology includes environmentally effective reduced-solvent methods. Among them SFE, ASE, or PLE and SWE, UAE, MAE, and solvent-free extraction are techniques of substantial significance

because they have opened a new route for producing and commercializing natural-based products through a totally automated in-line procedure. Recent breakthroughs in extraction landscape promote the replacement of classic solvents, such as *n*-hexane and chlorinated solvents, with alternative green extractants. *In silico* predictive methods, like conductor-like screening model for real solvents (COSMO-RS), quantitative structure–properties relationship (QSPR) and molecular modeling, constitute powerful tools because they can provide an accurate classification of industrial solvents and identify new extractants. Thus, according to computational characterization, the main categories of sustainable solvents are presented in Figure 3.3 [82]. Carbon dioxide ($CO_2$), water, glycerol, ionic liquids and natural deep eutectic solvents (NADES) are the ones commonly applied in high-energy extraction.

| Green Solvents | Characteristics | Examples |
|---|---|---|
| Supercritical solvents | (+): Nonflammable, nontoxic, inert, recyclable | $CO_2$, water |
| Biosolvents | (+): Derived from renewable sources | Natural acids derivatives (i.e., lactic acid), bioethanol, vegetable methyl-esters (i.e., methyl soyate), terpenes |
| Ecofriendly solvents | (+): Solvents that fulfills three green principals: environment-human-safety (ESH) | Succinic and adipic acid, 3-methoxy-3-methyl-butan-1-ol (MMB), glutaric dimethyl, diethyl and dibutyl esters |
| Liquid polymers | (+): Nonhazardous, nonvolatile, biocompatible; (−): Biodegradable | Polyethylene glycol (PEG), polydimethylsiloxane (PDMS) |
| Fluorinated solvents | (+): Nonhazardous, nonflammable; (−): Biodegradable | Perfluorooctanate ($C_8F_{18}$), perfluorotributylamine ($C_{12}F_{27}N$) |
| Ionic liquids/Eutectic mixtures | (+): Thermostable, nonvolatile; (−): Questionable toxicity | Imidazolium salts, choline acetate |

**Figure 3.3. Main categories of green solvents [73].**

However, in order to proceed with a complete replacement of decade-old extraction methods, a fruitful combination of analytical chemistry tools, updated validation protocols, sophisticated optimization statistical analysis and thorough surveys for economic feasibility are required [73].

## 3.3 Ultrasound-assisted (UAE) and Microwave-assisted extraction (MAE): Concepts and instrumentation

Over the last years, ultrasound (UAE)- and microwave-assisted extraction (MAE) are gaining widespread acceptance compared to classic liquid-solid extraction or other modern approaches.

### 3.3.1 Ultrasound-assisted extraction (UAE)

Over the last decades, ultrasounds (US) stand out as a powerful tool in food-processing industry, which shows more and more concern in protecting nutritional value, quality, and biofunctionality of nutraceutical products. US are mechanical waves of frequencies over 16 kHz, boundary for audible frequencies. Their application spectrum includes emulsification, homogenization, crystallization, low-temperature pasteurization, defoaming, dewatering, particle-size reduction, and changing viscosity, degassing, activation and inactivation of enzymes. However, UAE, first studied in the 1950s, has managed to intensify the extraction of a large number of bioactive ingredients by speeding up extraction rate and by augmenting extraction quality [83].

UAE theoretical background relies on acoustic cavitation phenomenon, which is based on contraction and rarefaction of ultrasonic gas bubbles incited on matrix molecules during the propagation of an ultrasound wave that penetrates it. During ultrasonication cycles, bubbles reach to a critical size, where they are no longer stable due to gas diffusion and they break down by releasing high amounts of energy and turbulence at extreme temperatures (~5000°C) and pressures (100 MPa). US intensity and energy plus vapor pressure, surface tension, temperature, and solvent viscosity, influence cavitation magnitude. The shear forces of microstreaming and microjetting effects produced by cavitation could facilitate solvent penetration for cell rupture and release of active compounds from matrix to extraction solvent. Categorized by frequency range, US technology is divided in two groups. High-frequency (100 kHz–1 MHz) low-power (<1 W cm-2) low-intensity US are normally taking part in quality assessment, such as physicochemical properties evaluation (acidity, firmness, sugar content, ripeness), as nondestructive technique. Low-frequency (16–100 kHz) high-power (10–1000 W cm-2) high-intensity is the type used in UAE applications [84]. UAE could reach to an extraction yield comparable to that of dated techniques within 10–90 min and could support higher sample throughput. Furthermore, this procedure is performed with generally recognized-as-safe (GRAS) solvents in relatively low processing temperatures, which cannot cause structural or functional alterations to analytes of interest.

Therefore, UAE is an ideal extraction type for an environmental safe and economically remunerative recovery of heat-sensitive molecules. Operational simplicity, high reproducibility, energy effectiveness, and high automatization level count up to the UAE advantages [85].

UAE instrumentation is quite simple as it only consists of the extraction medium, an energy generator and a transducer for energy conversion to its acoustic form. An indispensable apparatus in every laboratory is the ultrasonic water bath, which most of the times operates only at a two-frequency mode (40 and 56 kHz). In this case, sonication is indirect as acoustic waves need to be transmitted from the water to the sample through sample vessels. Present technological improvements deliver ultrasonic baths with multifrequency setup and also sonoreactors, cup, and microplate horns, which supply energies 50 times higher than baths in sealed matrix tubes. Nevertheless, a complete extraction of bioactive constituents from complex natural matrices requires more advanced and energy-boosting platforms. Ultrasonic probe (Fig. 3.4) is a state-of-art equipment in this field because it can provide high extraction yields within a few minutes due to the great amounts of energy (100-fold higher than water bath energy) that emancipate directly to extractant/matrix solution. Nowadays, companies' engineering departments manufacture different types of probes, such as titanium alloy probes, spiral probes, silica-glass probes, dual and multiprobes. In both instrumentations, temperature could be stabilized in controlled values. With ultrasonic probe, extraction intensity could be regulated in different intended levels [84].

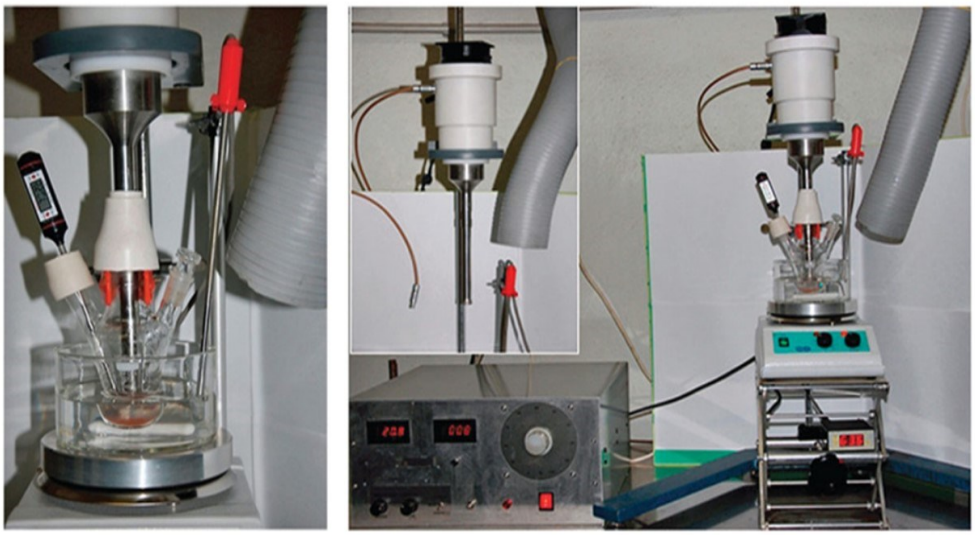


**Figure 3.4 UAE probe instrumentation (Sonics and Materials INC., Vibra-Cell VCX 750 (20 kHz, 750 W), ultrasonics processor, equipped with piezoelectric converter and 13 mm diameter probe fabricated from titanium alloy Ti-6Al-4V [73].**

### 3.3.2 Microwave-assisted extraction (MAE)

Electromagnetic waves with frequencies between 300 MHz and 300 GHz are accountable for MAE. MAE mechanism is based on microwave (MW) heating, which is produced by the synergistic combination of ionic conduction and dipole rotation. The physical principals behind these two phenomena are responsible for converting electromagnetic to thermal energy, which is transferred directly to the extractable material via molecular interactions of MW and polar solvents. Dielectric solvents or solvents with permanent dipole properties interact strongly with MW. Dielectric constant (ε), dielectric loss (ε″) and dissipation factor (tan δ) are estimators of solvents MW heating capacity. The aforementioned indices represent the ability of a solvent to absorb MW, to transform MW to thermal energy and to warm under MW, respectively. Higher dielectric constant and dissipation factor values indicate more sufficient MW absorption and higher extraction rates. Polar solvents, like water, which have high dielectric constant at lower dissipation value, could absorb amounts of energy higher than that it can deplete. The extreme induced temperatures provoke superheating effect inside the matrix that results to cell collapse, maximum diffusivity, and solute release into the extractant. As a typical high-energy extraction example MAE gathers all the advantages of these techniques (short extraction times, low solvent consumption and high extraction yields).

Although it can be used for thermolabile compounds extraction, MAE could be performed in temperatures near solvent boiling point and for that reason there is a possibility for compounds biofunctionality loss. Nonetheless, MAE could favor bioactive compounds quantitative isolation [86, 87].

Four major components constitute MAE devices, a magnetron or MW generator, a wave guide for MW diffusion, an applicator for samples, and a circulator for forward MW movement. Homogenous MW distribution is achieved by beam reflectors. Two different MAE types are developed for laboratory and large-scale applications. Closed vessel MAE is accomplished in controlled temperature and pressure, while open vessel or focused MAE is based on the solvent reflux and on sample-focused MW irradiation. Both MAE modes are available as single- and multimode devices. Closed vessel MAE is a pressurized system that requires less solvent volumes and operates in higher temperatures resulting in efficient recovery of volatile compounds. Despite higher yields, high pressures increase operational risk. Additionally, closed mode vials do not permit large amounts of material processing. Solvent addition during extraction and atmospheric pressure, make focused mode (Fig. 3.5) a more secure option. Instrumentation setup is simple and can be programmed to elaborate larger matrix quantities. Precision issues, lack of simultaneous sample processing, and longer extraction times are the main pitfalls of focused MAE [88]. Despite disadvantages, like extra filtration or centrifugation step, poorer recoveries of nonpolar or volatile compounds, and a risk of thermosensitive molecules deterioration, MAE is one of the most widely applicable high energy techniques due to its competitive advantages, such as more than 60% energy saving, compact systems, and high recoveries, compared to other methods [87].

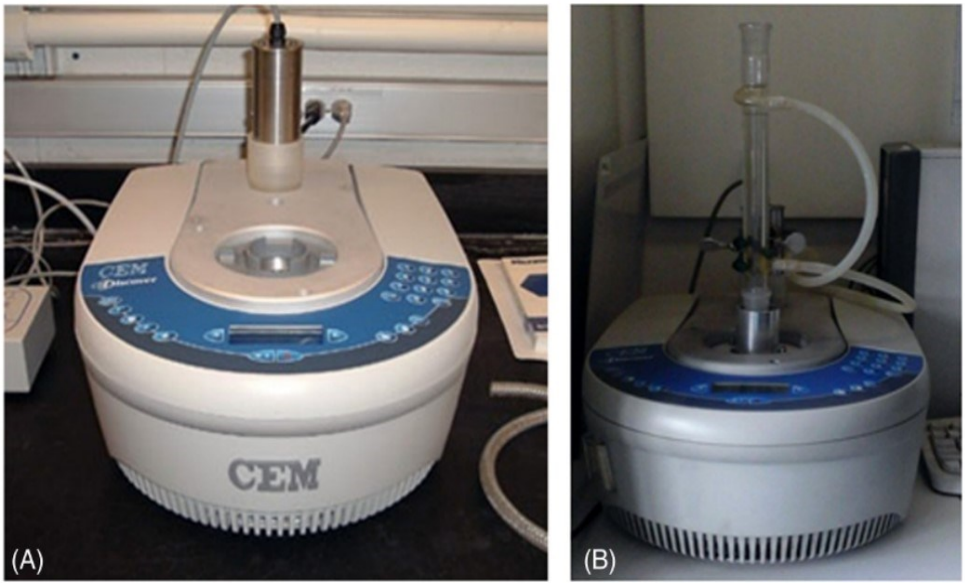



**Figure 3.5. Closed- (A) and open- (B) MAE instrumentation [73].**

As health and environment are liable to being harmed from industrial disposal of volatile organic extraction solvents, like *n*-hexane, a greener economically feasible answer is solvent-free microwave extraction (SFME). Under the umbrella of SFME fall techniques, such as pressurized SFME (PSFME), vacuum microwave hydro distillation (VMHD), improved SFME (ISFME), microwave steam diffusion (MSDf), and also versions of microwave hydrodiffusion and gravity (MHG), and microwave dry-diffusion and gravity (MDG) [89].

In general, SFME device includes a multimode MW reactor and an IR temperature sensor. Supplementary equipment is added according to SFME modifications [90] (Fig. 3.6). ISFME is an advanced SFME modification, which includes the addition of a MW-absorbing agent, that is, graphite and activated carbon powders. In VMHD, MW processing is combined with sequential vacuum implementation. MSD provides essential oils condensed extracts, while MSDf finds application in essential oils extraction from natural by-products. PSFME is a SFME at pressures higher than atmospheric. MHG is based on MW principals only in this method, cell rupture and release of analytes takes place without any solvent under thermal energy generated by MW at atmospheric pressure, a phenomenon called hydrodiffusion. Compounds' diffusion outside matrix is followed by gravity dropping out of MW reactor. Although MHG implementation is yet at an early stage, it catches scientific

world attention as a nonsolvent technique. A more sophisticated version of MHG, called vacuum MHG (VMHG) is already evolving. As matrix moisture is an essential component of MHG, dry samples could be processed by MDG [73, 89, 91]. Solvent-free high energy extraction exhibits tremendous possibilities for a wider nonpilot application as free-solvent procedures assist nonresidue and safer scale-up of these techniques by eliminating the risk of explosion and overpressure accidents.

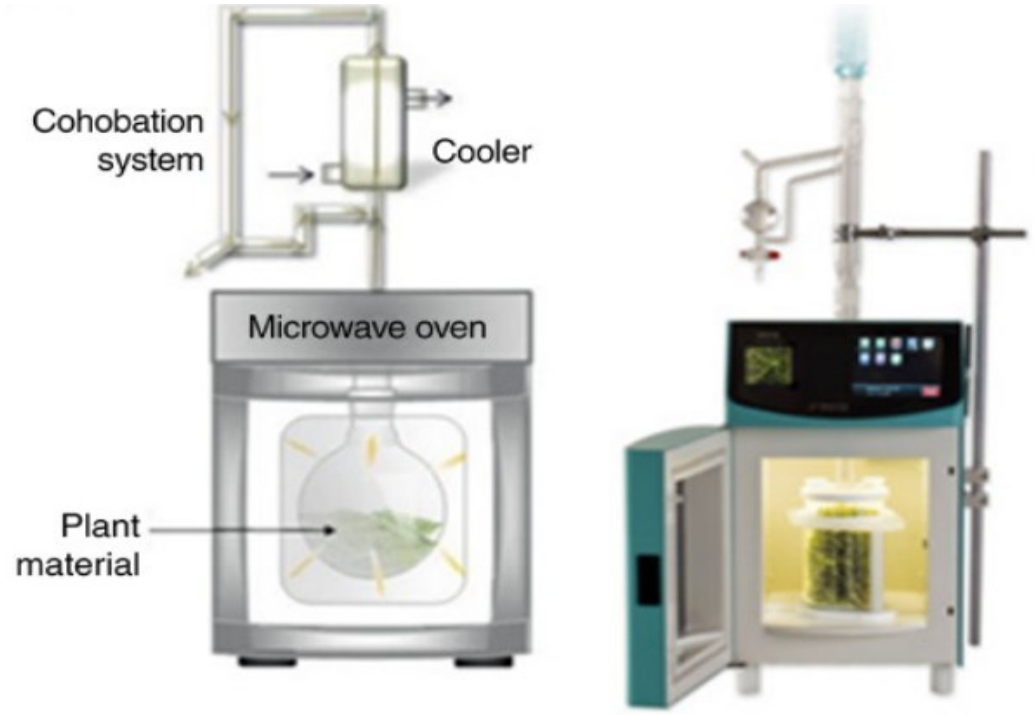



**Figure 3.6. SFME instrumentation [73].**

## 3.4 Ionic liquids (ILs)-Natural deep eutectic solvents (NADES)

As the demand for natural-based products marks an exponential ascent, an even more efficient scale-up of the existing extraction methods is top priority for massive production. A promising scheme toward this direction could be the synergistic combination of the already developed techniques in order to enhance extraction outcome by overcoming their implicit flaws.

Sustainability and eco-efficiency of large-scale processes is strongly supported by the replacement of hazardous organic solvents from ionic liquids (ILs) state-of-art technology, commonly known as molten salts. A vast number of salts that remain in their liquid phase at temperatures under 100°C due to their quiet large

size and high-molecular asymmetry constitute the group of ILs. Their intriguing characteristics arise from their pure ionic nature, which is formed from organic cations (i.e., ammonium, pyridinium, imidazolium phosphonium, pyrrolidinium) and both inorganic ($Cl^-$, $PF_6^-$, $BF_4^-$) and organic anions (trifluoromethylsulfonate $[CF_3SO_3]^-$, *bis*[(trifluoromethyl)sulfonyl]imide $[(CF_3SO_2)_2N]^-$, trifluoroethanoate $[CF_3CO_2]^-$). Their ionic character is responsible for their advantageous properties, for example, ecoviability, thermal stability, almost zero vapor pressure and low volatility, electrolytic conductivity, flexibility in viscosity and miscibility values and liquid types, ability for fit-to-purpose IL synthesis, recycling and reuse, inflammability, distinct physicochemical properties based on cation–anion combination, and coupling with almost every known analytical technique [92, 93].

Deep eutectic solvents (DESs), which are naturally composed multimixtures between hydrogen bond donors and hydrogen bond acceptors with lower melting points than their forming components, are also a breakthrough in the green substitution of classic volatile solvents because they are biodegradable, cheap, easily made mediums. DES examples are urea mixtures (choline chloride-urea, 2:5 mixture of L-proline and glycerol), quaternary ammonium halide salts and phosphonium halide salts [94, 95].DESs possess a great number of interesting properties, such as low vapor pressure and high viscosity, which make them suitable media for various processes, including extraction of natural compounds [96, 97].

Recent studies have been focusing on a subcategory of DESs, the Natural Deep Eutectic Solvents (NADESs), whose ingredients are naturally occurring chemical compounds. These solvents except of the above mentioned properties are also characterized of low or no toxicity and high biodegradability potential, which are two of the main aspects of green chemistry [98]. NADESs are considered as promising solvents for extraction processes, due to the formation of intense hydrogen bonds between the NADES components and the extracted compounds, which increase the process yield and stabilize the obtained extract, thus protecting the molecules from degradation [99]. Replacing conventional solvents with NADES and hyphenating them with modern high energy extraction methods, like ultrasound- (UAE) and

microwave-assisted extraction (MAE) improves extraction yield, reduces extraction time, compounds' degradation and adverse ecological effects [73, 100].

Despite the fact that both technologies are still in their early stages, they are considered among the most auspicious suggestions for viable extraction scale-up of bioactive components as they have incorporated in classic liquid extraction and microextraction [101, 102]. They are also effectively hyphenated with UAE, MAE or platforms of multiple high energy techniques, that is, UAE-MAE-ILs as shown in Table 3.1.

**Table 3.1. Examples of green solvents implementation to UAE and MAE.**

| Technique | Matrix | Target compounds | Reference |
|---|---|---|---|
| ILs-UAE | Fruits | Lignans | [103] |
| | Rosemary | Carnosic acid-Rosmarinic acid | [104] |
| | Shrimp waste | Astaxanthin | [105] |
| ILs-MAE | Lotus leaves | Alkaloids | [106] |
| | Rosemary | Carnosic acid-Rosmarinic acid-EOs | [107] |
| | Pigeon pea leaves | Flavanones-Stilbenoids- | [108] |
| ILs-UAE-MAE | Burdock leaves | Phenolic compounds | [109] |
| | *Galla chinensis* herb | Tannins | [110] |
| DES-UAE | Sheep, bovine, chicken liver | Metals | [111] |
| | Shrimp by-products | Astaxanthin | [112] |
| | *Flos sophorae* herb | Quercetin-Kaempferol-Isorhamnetin glycosides | [94] |
| | Grape skin | Phenolics | [113] |
| DES-MAE | Pigeon pea roots | Genistin-Genistein-Apigenin | [114] |
| | *Radix Scutellariae* plant | Flavonoids | [115] |
| | Grape skin | Phenolics | [113] |

### 3.5 Coupled high energy techniques

Since Soxhlet extraction still stands as a reliable approach for in-factory extraction, there is a serious attempt for modern modifications in order to cut down extraction time and surpass recurring disadvantages. High-pressure Soxhlet extraction simulates to SFE. Although solvents do not reach their critical conditions, they preserve their liquid character in high pressures (∼7–10 MPa). Automated Soxhlet devices combine a dual-mode extractor with Soxhlet heating and a reflux system. UAE benefits could be integrated to Soxhlet extraction, when a US probe offers energy through a thermostated water bath, where Soxhlet flask is immersed. Anyhow, Soxhlet-MAE coupling is the most commercially feasible platform. In Soxhlet-MAE setup, extraction is ensued at atmospheric pressures and focused MW mode, while extraction basis is established on Soxhlet principals [116]. Although these combinations have not yet caught the eye of industrial community, faster process, enriched final extracts, lower solvent consumption, automation, extraction cycles reduction, and quantitative recoveries are the highlights of Soxhlet-high energy methods hyphenation [117].

Apart from novel extractants and Soxhlet-based modernized techniques, the combination of high-energy methods in a single-step process or in a sequential continuous procedure empowers the positive effects of individual techniques. Lab studies worldwide have deployed equipment and principals of US and MW (UMAE) [118-121], US and SF (UAE-SFE or UASFE) [122-127], and MAE-SFE [128], UAE-DLLME [129-131], and solid phase (SPE)-high energy extraction [132] for natural products recovery. UMAE results to faster heating due to MW energy and more efficient analyte release due to US effect. UASFE higher yields are attributed to improved mass transfer due to US cavitation and diffusion impact on SF properties. DLLME is coupled with MAE and UAE usually for extracting and determining metal, amines types, and hydrocarbons. SPE conjoining in extraction layout contribute to sample cleanup and preconcentration and therefore it enhances procedure selectivity by reducing at the same time total analysis steps. SPE is more adaptable to ASE since the use of organic pressurized solvent activates better the solid phase material. Howbeit, one of the greatest achievements of combined high-energy methods

is the acceleration or initiation of targeted chemical reactions, that is, hydrolysis concurrently with the extraction process. Notwithstanding, further studies concerning the increased number of critical extraction factors and system complexity is a vital action for commercialization of coupled techniques [133]. Typical examples of combinatory/hyphenated extraction are exhibited in Table 3.2.

**Table 3.2. Coupled high energy techniques.**

| Technique | Matrix | Target compounds | Reference |
|---|---|---|---|
| MAE-Soxhlet | Ginseng | Pesticides | [116] |
| UMAE | Tomatoes | Lycopene | [120] |
| | *Inonotus obliquus* fungus-*Tricholoma mongolicum Imai* mushroom | Polysaccharides | [118, 121] |
| | *Gelsemium elegans* plant | Indole alkaloids | [128] |
| UASFE | *Capsicum* peppers | Phenolics<br>Capsaicinoids | [122, 123] |
| | Algae | Isoflavones | [124] |
| | Tea | Caffeine | [125] |
| | Clove buds | Oils | [126] |
| | *Scutellaria barbataD. Don-Hedyotis diffusa* plants | Oleanolic and ursolic acid | [127, 134] |
| | Blackberry bagasse | Antioxidant compounds | [135] |
| MAE-SFE | *Gynura Segetum* plant | Alkaloids | [128] |
| UAE-DLLME | *Oliveria decumbens* Vent. | EOs | [131] |
| | Saffron-Tea plants | Volatiles | [129, 130] |
| | *Edgeworthia chrysantha* Lindl flower | EOs | [136] |
| UAE-SPE | Pharmaceutical samples | Thymol and carvacrol | [132] |

## 3.6 Experimental Design (DOE): In the spotlight of optimization strategies

The alpha and omega of industrial online production is the optimization of each procedure step in order to support a viable remunerative outcome. Classic optimization approaches, like one variable-at-time (OVAT), a univariate method where each factor is optimized individually by keeping the rest of the parameters at a predefined constant value, are an obsolete experimental planning. OVAT unsuitability is ascribed to the large number of experimental runs needed, to the lack of estimating the interactions between the experimental factors and the deficiency of determining the global optimum since the acquired data only provide information about the points where the experiments were conducted [137].

Instead of applying this underachieving approach, laboratories, and corporations use multivariate optimization strategies, known as experimental design (DOE) in order to improve effectiveness. These statistical tools allow the simultaneous optimization of more than one experimental parameter or independent factor at several predefined value levels and estimation of their interactions effects on more than one experimental response or dependent factor by performing the minimum necessary number of experimental runs. Built on mathematical algorithms and transformations, DOE supplies accurate, reproducible, and validated models through the interpretation of statistical indices ($R^2$, $R_{adjusted}^2$ , mean square (MS), ANOVA-test, *p*-value, lack of fit), charts and plots (Pareto chart, predicted vs. experimental value charts, contour plots, 3D surface plots) [138].

Usually, a DOE setup consists of two sequential designs. Screening models, for example, two-level full or fractional factorial ($2^{k-n}$, where 2 applies to the number of value levels, *k* to the number of experimental independent factors and *n* to the size of fraction used from full factorial matrix) and Plackett–Burman designs, are applied for a preliminary screening of the experimental variables and their values aiming at finding the parameters with crucial effects ($p$-value ≤ 0.05) on the response and confining the range of near-to-optimum experimental values. Both full and fractional factorial designs could identify major parameters and their interactions effect, while Plackett–Burman could estimate only the

important main effects of numerous factors by performing really few experimental runs. Therefore, this model is basically used for liquid/gas chromatography-mass spectrometry (LC/GC-MS) optimization purposes. On the other hand, factorial designs can be applied when the number of experimental variables is less than 16 because multiplying factors numbers increase significantly total experimental runs. Concerning extraction methods, full factorial designs are highly recommended as by reckoning in all interactions terms, a more reliable model could be produced [139].

Screening designs conclusions point out the right direction of optimization models, otherwise noted as response surface models (RSM) in terms of important factors and value range. RSM are three level-minimum models, adequate for determining the exact optimal conditions of a process. The most popular RSM designs are symmetrical three-level full factorial, central composite (CCD), Doehlert and Box–Behnken (BBD) designs, asymmetrical D-optimal and Kennan-Stone algorithm designs and mixture designs, like the three-component simplex lattice design used for solvent mixture composition optimization [138]. CCD and BBD are the most ubiquitous models in extraction applications. Each step of a DOE flowchart is illustrated in Figure 3.7.

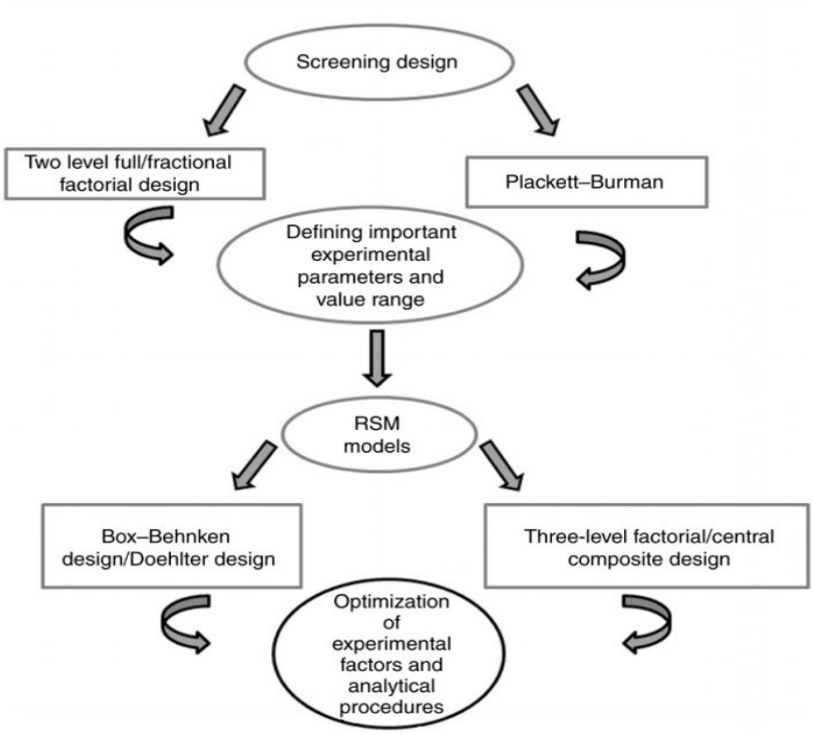



**Figure 3.7. Flowchart of DOE [73].**

## 3.7 Optimizing extraction parameters by applying DOE

In high-energy extraction field, DOE is applied for optimization of various critical parameters illustrated in Figure 3.8.

| Experimental Parameters | SFE | ASE-SWE | UAE | MAE |
|---|---|---|---|---|
| Pressure | + | + | – | ±* |
| Temperature | + | + | + | + |
| Extraction solvent system | + | + | + | + |
| Extraction time | + | + | + | + |
| Energy power | – | – | + | + |
| Solvent/material ratio | + | + | + | + |
| Particle size | + | + | + | + |
| Moisture content | + | + | – | + |
| Solvent flow rate | + | + | – | – |
| pH | – | + | – | – |
| Modifiers addition | – | + | – | – |
| Extraction mode | + | + | – | + |

+, Closed-vessel mode; –, open-vessel mode.

**Figure 3.8. Common extraction parameters optimized by DOE [73].**

More explicitly, higher pressures are applied for keeping solvent in the liquid form and for facilitating the traversal of the analytes through matrix pores. Elevated temperatures increase mass transfer and compound solubility, but they may affect negatively extraction selectivity. Especially for bioactive molecules extraction, temperatures less than 100°C should be preferred due to compounds thermosensitivity [85, 140].

Extraction solvent systems depend crucially not only on the group of extracted compounds and its affinity to the solvent, on the extractant properties, like polarity, density, boiling point, and toxicity but also on the principals of each extraction method [141]. In UAE, acoustic cavitation is intensified when extraction solvents are liquids with low viscosity, which enable cell penetration, and low vapor pressure. Notwithstanding, low vapor pressure solvents could develop locally really extreme pressure and temperature causing molecule degradation. Thus, medium vapor pressure solvents with high-molecular affinity to the target molecules are selected [142, 143]. MAE extraction solvent systems include solvents that absorb MW energy, mixtures of solvents with high- and low-dissipation factors and MW transparent solvents with matrices of high

dielectric loss. Among them, mixtures of low and high absorbing ability, like hexane and ethanol, give better yields because their heating is milder and varies according to their exact composition. Facing the possible deterioration of heat-sensitive components, use of very absorbing MW solvents (i.e. water) leads to shorter exposure times, but in general, longer duration renders better results [88].

Extraction time effect is almost similar to that of temperature. Although, efficient yields are obtained in very short exposure times, higher extraction periods provide higher extraction yields. For instance, in UAE with ultrasonic probe optimal extraction time is less than 30 minutes. However, optimal extraction time should be a compromise between extraction yields and compounds susceptibility to thermodegradation [88, 120]. Maximum mass transfer, solvent diffusion, and matrix disruption is achieved normally at higher (UAE) or with a succession of low and moderate power (MAE) and at higher solvent/material ratio.

While higher solvent/material ratios increase extraction yields, they could extend analysis time by adding up extra steps, like solvent evaporation [88, 144, 145]. Smaller matrix particle size maximizes contact surface between substrate and solvent and thus leads to higher extraction recoveries. Higher flow rates reduce target compounds dwelling time in high temperatures and therefore side reactions. Since flow rates affect significantly analyte kinetics, their optimal value should be established in order to improve extraction yield. On the other hand, an optimal flow rate controls overdilution of compounds of interest that could take place in excessively high flow rates [43].

Modifiers addition is commonly recommended in ASE cases. Strong acids and bases along with fluids with autoignition temperature of 40–200°C (carbon disulfide, diethyl ether, and 1,4-dioxane) cannot be used in ASE. Addition of inert modifiers, such as hydromatrix, sodium sulfate, quartz sands, and basic alumina, could be essential to extraction efficiency improvement due to better solute dispersion. Uniform dispersion could be also achieved with agitation that blocks agglomerates formation. Organic and inorganic agents improve solubility by enhancing analyte–solvent interactions [140].

Parameters with minor effects that also need to be set to predetermined values for higher extraction yields, especially in the case of more automated techniques like SFE and ASE, are preheating time (~5 min), flush volume for moving away all analytes before new extraction cycle (~60%), and purge time. Optimization strategies should also be applied in order to amend extraction kinetics (i.e., analyte desorption from substrate, analyte diffusion into the matrix, solute dissolution into the solvent, and target compound elution) [43, 140]. Normally, a pulsed sonication in UAE is preferred from a continuous mode for energy-saving reasons. A zero-ramping time value in MAE reduces extraction time without any significant losses of target compounds [146].

Extraction mode is pretty much a deciding factor when we refer to ASE and MAE. Regarding ASE, even though extraction conditions in static mode are stable, high-concentrated samples, or low solubility analytes could be recovered completely if extraction process is repeated several times. A faster and better extraction recovery could be ensued in dynamic mode due to the variations in extraction process but higher solvent volumes are an obstacle for its wide application. A static-dynamic extraction mode could be an optimal solution in terms of profit since it combines lower solvent volumes of static extraction and shorter extraction times of dynamic mode. By succeeding more intense extract suspension in shorter periods, the dual model eludes solute degradation and results to higher extraction yields. In MAE, open-vessel mode is the more applicable version because it is compatible with a wide range of extracted molecules with the exception of volatile components, which are generally extracted by closed-vessel systems to avoid possible losses [88, 140]. Overall, the same group of bioactive molecules could necessitate totally different extraction conditions depending on substrate nature and optimal conditions could differ according to the aim of the process. In some cases, extraction objective is higher yields and in others, maximal bioactivity of extracted compounds. Some of the most recent indicative examples of DOE models used in high energy extraction optimization include two-level full or fractional factorial, Plackett-Burman, three-level factorial, central composite (CCD), Box-Behnken, Doehlert and D-optimal design [73].

## 3.8 Carotenoids recovery: A high energy-oriented approach for extracting them from natural sources

Since high-energy extraction techniques are a fast eco-compatible solution, which processes 'layout require lower solvent quantities and provide quantitative extraction yields without any special pretreatment, they are gaining momentum in biofunctional compounds' extraction field. It will only take a few minutes for someone to find out that the number of peer-reviewed reports related to high-energy extraction of biofunctional molecules has been soared especially the last 5 years. Their application range broadens as these methods shield molecules susceptible to light and oxygen degradation. They could also be a money- and time-saving alternative for extraction of polyunsaturated and monounsaturated FAs, neutral and polar lipids, vegetable and fish oils, triglycerides, EOs, phenolic compounds and polyphenols, tannins, anthocyanins and flavonoids, carotenoids and other natural pigments, vitamins and terpenoids, sterols and polysaccharides, capsaicinoids, alkaloids and curcuminoids from almost every type of natural sources, from spices and plants to marine organisms and algae (macro- and microalgae, cyanobacteria, invertebrates, crustaceans) [147-149].

High energy alternative is nowadays the number one option for extracting carotenoids [150]. Contrary to animal and plant organisms, algae can produce high amounts of carotenoids, therefore [151, 152] considered *Neochloris oleoabundans* and *H. pluvialis* microalgae as suitable matrices for SFE of carotenoids, especially astaxanthin. Nevertheless, SFE is also a handy solution for carotenoids recovery from pink shrimp [153], pumpkin [154] and pigmented rice bran [155].

By applying ASE [156] delivered the carotenoid profile of 27 types of red, yellow, and orange paprika based on their shapes and cultivation type An LC-MS profiling was carried out in ASE carotenoid extracts from Tunisian kaki, apricots, and peaches [157]. Heo et al. (2014) succeeded the separation of the isomers lutein and zeaxanthin by ASE and UPLC [158].

Carrot and tomato waste are two of the most opportune substrates for extraction high yields of carotenoids [159] recovered carotenoids from tomato

pomace by combing moderate pressure with UAE and Purohit and Gogate (2015), [160] investigated the use of vegetable oils as UAE solvents for b-carotene from carrot residues. An NMR assay was performed by Sobolev et al. (2014) [161] in MAE extracts of saffron for structural elucidation of crocin, crocetin, and picocrocin. As shown in the work of Hiranvarachat and Devahastin (2014) [162], a non-continuous MW irradiation enhances the antioxidant capacity of carrot peels carotenoids extracts. In order to improve b-carotene extractability Hiranvarachat et al. (2012) [163] tested the effect of different pretreatment procedures, like soaking in citric acid and blanching in water and citric acid. Ho et al. (2015) [164] isolated *cis*- and *trans*-lycopene in tomato peels MAE extracts. Tomato paste was also used as substrate in a Lianfu and Zelong (2008) [120] project for a comparative study between UAE and UMAE, where UMAE resulted in higher yields. Roohinejad et al. (2014) [165] and Luengo et al. (2014, 2015) [166, 167] lab groups used PEFE for extracting carotenoids from carrot by-products and microalgae *C. vulgaris*, respectively.

### 3.9 High energy extraction and metabolomics

Metabolomics is extensively used to study metabolites composition of biological samples and natural products as these are influenced by extraction, production and/or processing methods in order to establish and predict their identity and activity. In view of the complexity of natural matrices, NMR- and MS-based metabolomics are considered the most promising techniques for matrix metabolites profiling. NMR-based metabolomics has been previously reported as a robust, quick, reproducible and non-destructive tool which can simultaneously identify diverse groups of abundant primary and secondary metabolites. On the other hand, MS-based metabolomics provide highly accurate and sensitive measurements of thousands of biomolecules in one single run [168, 169].

In particular, plant metabolomics is the answer to the traditional phytochemical approaches, which are focused on the analysis of specific targeted metabolites, usually a group of bioactive compounds (i.e. carotenoids, polyphenols, alkaloids, amino acids) and not on the complete and detailed metabolic profile of the plant substrate or plant byproducts. However, the different composition of the plant matrix, any possible enzymatic degradation or chemical breakdown

of plant metabolites and the lack of a tailor-made validated extraction step affects crucially the final quality of the metabolomic study and the number of identified metabolites [170].

Among these factors, the step of sample preparation is of utmost importance since the information provided afterwards from high throughput analytical techniques is highly dependent on the selected extraction method. Thus, special attention should be paid to the development of a comprehensive extraction methodology that will investigate as exhaustively as possible the plant metabolome [170].

Until recently, classic extraction were the method of choice in the majority of –omics studies [171-173]. Most of the times, traditional extraction methods are based on the previous experience and knowledge of the researchers, who apply them without further optimization or validation. In parallel, high energy extraction methods are usually ignored or sub exploited in plant metabolomics studies. However, these methods could be superior to classic extractions in terms of cost, efficiency, fractionation ability and time. Therefore, the capitalization of DOE-high energy strong points and their incorporation to plant metabolomics merits further investigation in order to generate a new robust and reliable sample preparation step for metabolomics studies [174].

# CHAPTER 4
# AIMS AND OBJECTIVES OF THE THESIS

Considering the importance of carotenoids as a novel and promising nutraceutical and cosmeceutical trend, the current work aims to create an integrated analytical platform based on high energy extractions in order to evaluate and determine the extraction conditions that result in different natural extracts of high carotenoid content. Furthermore, the up-to-date traditional extraction methodologies are pretty much based on targeted approaches that provide limited and skewed data about the complete metabolome of the plant substrates. Thus, this study attempts to bridge the gap between extraction strategies and plant metabolomics by designing a comprehensive validated extraction protocol in order to access information not only for the targeted (carotenoids) but also for the untargeted metabolites of different DOE-fractions.

In particular, the main objective of the present project is **to highlight and promote ways to valorize an important local agro-industry byproduct (apricot pulp) and a marine byproduct (shrimp head) through their transformation to high-added value products.**

Furthermore, other additional goals ensuing from this thesis are as follows:

1) Implementing modern innovative techniques (UAE and MAE) and solvents (NADES) in order to overcome classic methods' drawbacks and to determine the most efficient extraction strategy for each substrate, either byproduct or food product.

2) Optimizing the targeted recovery of carotenoids from the investigated natural substrates using DOE models.

3) Performing a qualitative and quantitative analysis of carotenoids extracts using a newly developed and validated APCI(+) LC-PDA-MS/MS method.

4) Compare different extraction conditions using NMR-based metabolic profiling of DOE-UAE/MAE fractions of apricots pulp and evaluate extracts according to co-extractants.

5) Upgrading the up-to-date sub-exploited utilization of high energy techniques in the field of foodomics.

By meticulously reviewing the current published works, we couldn't help noticing the omission and limited data regarding the implementation of validated high energy extraction methods to the metabolomics field for the study of untargeted plant metabolites. Therefore, the integration of high energy extraction methods and 1H-NMR-based metabolomics to point out putative bioactive components of different DOE extracts from agro-byproducts is of utmost importance. Moreover, going through the current literature, we noticed that there is a circumscribed number of published papers regarding the extraction of non-polar carotenoids (i.e. b-carotene) or xanthophylls (astaxanthin, lutein, etc.) using eco-compatible NADES as extractant media. In addition, the present study is aiming to examine the lipidic effect of natural substrates on carotenoids extraction through the quantitation of carotenoids in lipid matrices. Hence, **the purpose of this work is to introduce a complementary analytical platform by merging modern extraction approaches and high throughput analytical tools (i.e. LC-MS/MS analysis, NMR metabolomics) and provide an alternative approach for the future scaling-up of carotenoids recovery from agro-byproducts and fat-rich food matrices.** The prospective successful adaption of the developed methodology to large-scale processes will open the way for the formulation of novel nutricosmetics, cosmeceuticals and nutraceutical products (dermal products, food supplements, eye drops, capsules and sprays). The steps of the present combinatorial analytical procedure are presented in Figure 4.1.

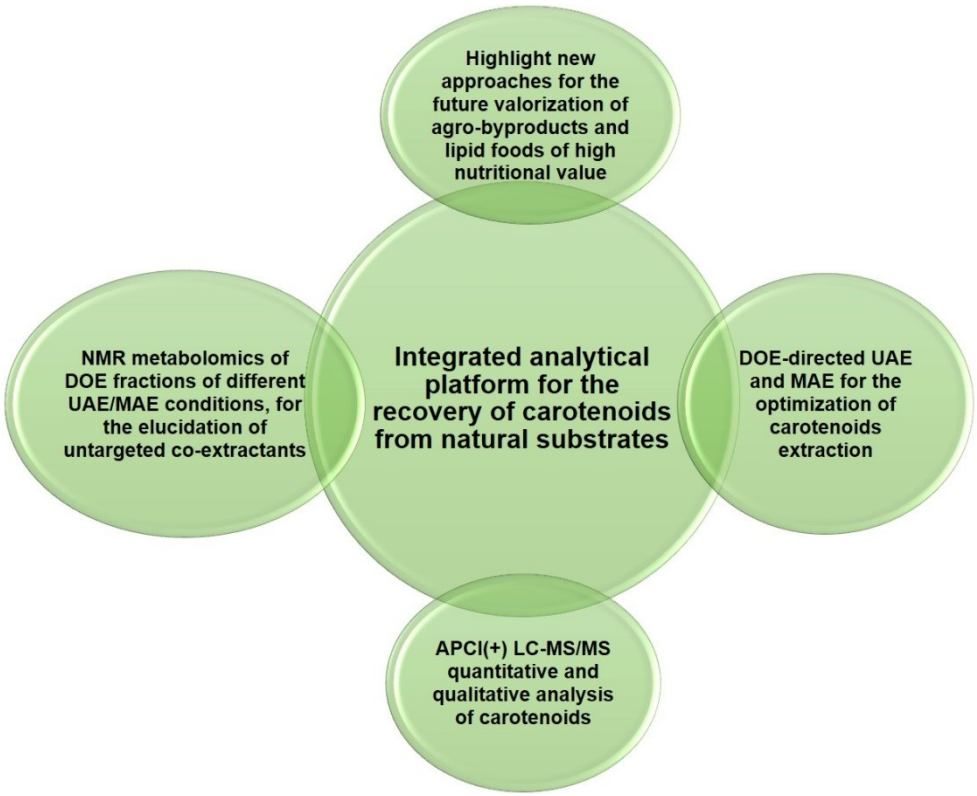



**Figure 4.1. Flowchart of the developed analytical platform.**

# CHAPTER 5
# MATERIALS AND METHODS

## 5.1 Reagents and standards

Beta-carotene (b-carotene) and *trans*-β-apo-8′-carotenal were purchased from Sigma-Aldrich (St. Louis, USA). *trans*-Lutein and *trans*-zeaxanthin were acquired from Extrasynthese (Genay, France), while *trans*-canthaxanthin and *trans*-astaxanthin were obtained from Fluka (St. Louis, USA).

All solvents tested were of analytical grade. Acetone was purchased from ChemLab (Zedelgem, Belgium) while chloroform, methanol, ethanol, N,N-dimethylformamide (DMF), isopropanol, petroleum ether and *n*-hexane were obtained from Merck (Darmstadt, Germany) and Scharlau (Barcelona, Spain). LC-MS grade methanol, acetonitrile and methyl-*tert*-butyl ether (MTBE) were provided by Fluka (St. Louis, USA) and Fischer Chemical (Pittsburgh, USA). Choline chloride and L(+)-tartaric acid were obtained by Fluka (St. Louis, USA).

All extracts and standard stock solutions (100.0 μg $mL^{-1}$) were kept in the freezer at -20ºC. Carotenoid standard working solutions of different concentrations were prepared daily by the appropriate dilution of carotenoids standard stock solution.

## 5.2 Sample-set and sample treatment

Apricots pulp was kindly provided by Danais S,A Fruit Processing Industry & Export Company (www.danais-sa.com). Apricot fruits of 'Bebekos' variety were collected from the region of Argos, Peloponnese, Greece during June 2017. Apricot byproducts, which were generated during the processing and compression of raw fruits, included mainly skin and also flesh of particle size over 0.5 mm. The average weight of apricots pulp samples were 17.1 g±2.1, n=10.

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 [175]. Egg yolks were separated *per manus* from eggs and were weighed (12.7 g±2.9, n=10).

Specimens of red shrimps, *Aristeus antennatus* were caught during spring of 2014 from Aegean Sea area and were purchased from a commercial fish market. The specimens (3 batches of 10 kg) were transported to the laboratory where they were washed with cold distilled water, weighed (41.5±4.9 g, n =10) and the average length were measured (16.30±0.67 cm, n =10). Then, the specimens were divided in six groups according to their weight and then were dissected.

Raw samples of all substrates were freeze dried in a ModulyoD Freeze Dryer, equipped with a Thermo Savant ValuPump VLP200 (Thermo Electron Corporation, Thermo Fischer, USA). Freeze-drying was selected as drying method since it protects sensitive metabolites for degradation during long-term storage. This method removes sample moisture that may produce undesirable chemical reactions and promote microbial growth [176]. Lyophilization is also highly recommended for carotenoids extraction as water content of substrates hinder the recovery of non-hydrophilic compounds (i.e carotenoids) from them [6]. Dried material was homogenized and powdered in a laboratory mill (Type ZM1, Retsch GmbH, Haan, Germany). Dry material were kept in glass jars at -20°C.

### 5.3 Extraction instrumentation

UAE process was performed 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–4V. 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.

### 5.4 Natural deep eutectic solvent (NADES) synthesis

Choline chloride/Tartaric acid (CC/TA) solvent was prepared using the heating method as described below: Choline chloride and tartaric acid in a molar ration 2:1 were mixed and stirred at 80 $^{o}$C for 4 h under inert atmosphere. Both choline chloride and CC/TA were dried in high vacuum before use for 3 h [93, 177]. The

molecular structure of CC/TA mixture is presented in Figure 5.1.

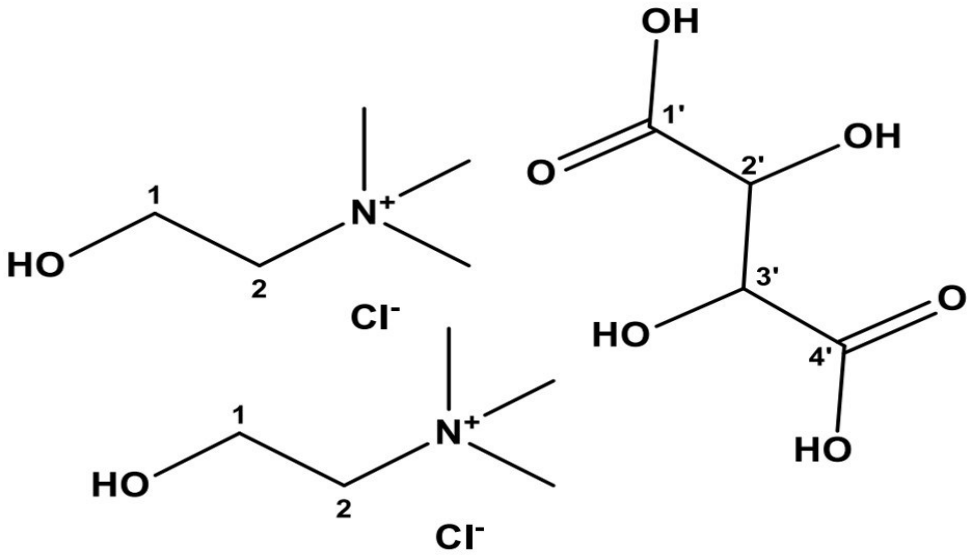



**Figure 5.1. Molecular structure of CC/TA solvent [177].**

### 5.4.1 NMR characterization

Choline chloride/Tartaric acid (CC/TA) was characterized by 1H, NOESY and COSY NMR on a Varian-600 MHz NMR spectrometer (Varian, USA). Briefly, 20 mg of dried CC/TA were dissolved in deuterated dimethyl sulfoxide (DMSO-$d_6$) and this solution was used for the NMR experiments [177].

The assigned peaks for the characterization of CC/TA solvent are shown below.

1H NMR (25 ºC, 600 MHz, DMSO-$d_6$) δ (ppm): 4.31 (2H, singlet, -CH tartaric acid), 3.8 (4H, broad signal, $(CH_3)_3$-N-$CH_2$), 3.44 (4H, triplet, J = 5 Hz, $CH_2$-$CH_2$-OH), 3.15 (18H, singlet, 6x$CH_3$) [177].

### 5.4.2 Thermal properties

Thermal properties of the CC/TA as well as the physical mixture of the individual components were studied using Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC). In both cases, the experiments were conducted from 20-500 ºC, with temperature rising in a rate of 5 ºC $min^{-1}$, under inert $N_2$ atmosphere [177].

## 5.5 Extraction processes

Three grams (3 g), for UAE, and one gram (1 g), for MAE, of dried apricot, egg yolk, shrimp head and body powder were suspended in various volumes of

different extraction solvents. Methanol, ethanol, chloroform, *n*-hexane, acetone and their mixtures were the solvents tested in apricot byproducts extraction. Chloroform, methanol, *n*-hexane, acetone and mixtures of various combinations of the aforementioned solvents were used in egg yolk extraction. Acetone, petroleum ether, *n*-hexane, isopropanol, DMF and ethanol were shrimps extraction solvents [6, 178]. After UAE or MAE, extracts were centrifuged at 8000 rpm for 20 minutes.

For classical extraction, used as a reference method for comparison purposes, the procedure was performed with 12:1 (v/w) solvent/material ratio and according to a modified 3-step Folch method [179]. 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.

A rotary evaporator at 50°C was used for acquiring dry residues of extracts. The obtained lipid fraction was diluted in 8 mL of extraction solvent. Next, volume aliquots of carotenoid extract were flushed with $N_2$ stream in order to obtain dry residues of 20 mg of apricots extract, 100 mg of egg yolk extract, 40 mg of shrimp head and 20 mg of shrimp body extract. Lastly, $N_2$ dry residues were dissolved in certain final volumes of acetone (for *vis*-spectrophotometry) or methanol:MTBE 1:1 v/v mixture (for LC-PDA-MS/MS) or deuterated solvents (for NMR). The flowchart of extraction process is illustrated in Figure 5.2.

Ultrasound-assisted (UAE) and microwave-assisted extraction (MAE) using NADES was carried out using a 80:20 CC/TA-MeOH mixture as extraction solvent. Since carotenoids are lipophilic molecules, NADES was combined with MeOH instead of $H_2O$ in order to increase their solubility in final solvent system [180, 181]. Three grams (3 g), for UAE, and one gram (1 g), for MAE, of lyophilized samples were suspended in different volumes of extraction solvent.

After each extraction process, the solid material was separated from the supernatant by centrifugation at 15000 rpm for 20 min. An aliquot of NADES-MeOH extracts was kept at -20 ºC until further use.

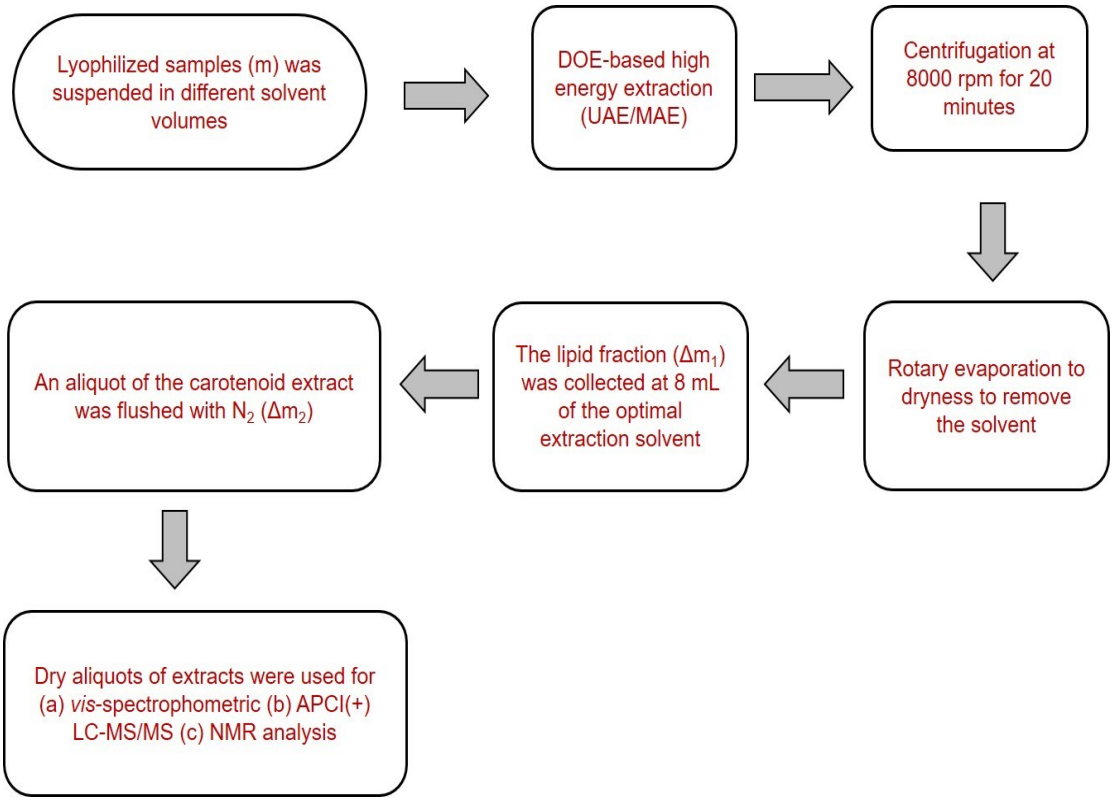



**Figure 5.2. Flowchart of the experimental process.**

## 5.6 Spectrophotometric estimation of extracts' total carotenoid content

Total carotenoid content of extracts was determined using a double-beam UV-*Vis* spectrophotometer Hitachi U-3210 (Hitachi Ltd., Tokyo, Japan). The wavelength screening (380-600 nm) for determining maximum absorbance wavelength of carotenoids provided the spectra presented in Supplementary data, Figure S1. As it is obvious, spectra of b-carotene, lutein and zeaxanthin (Fig. S1a-c) and spectra of astaxanthin and canthaxanthin (Fig. S1d-e) were almost identical, therefore carotenoid content of extracts was expressed in equivalents of each substrate's major carotenoid and its maximum wavelength.

Specifically, apricot byproducts' carotenoid content was calculated at 455 nm, maximum wavelength of b-carotene and expressed in b-carotene equivalents at concentration range of 0.25-7.5 μg $mL^{-1}$ since this is the main carotenoid of apricots [29]. The analytical figures of the calibration curve of absorbance (A)

versus b-carotene concentration (C) was: A=0.1436(±0.0013)C-0.0038(±0.0050), $R^2$=0.999 (Equation 1).

Spectrophotometric determination of egg yolk carotenoid content was carried out using lutein calibration curve at 447 nm [182]. The concentration range of lutein standard solution was 0.10–7.5 μg $mL^{-1}$. Equally, the analytical figures for the calibration curve of absorbance versus lutein concentration was: A=0.2200(±0.0041)C+0.016(±0.014), $R^2$=0.998 (Equation 2).

Total carotenoid content of shrimp extracts was determined at 478 nm by the spectrophotometric calibration curve of absorbance versus astaxanthin concentration, which is over 75% of the total carotenoids in shrimps [183]. The concentration range of astaxanthin standard solutions was 1-10 μg $mL^{-1}$. The analytical figures of this calibration curve was: A=0.1670(±0.0057)C+0.009(±0.033), $R^2$=0.994 (Equation 3).

Acetone was used as the solvent of all samples and as the blank sample. All experiments were conducted in triplicate. The extraction yield of total carotenoids was calculated according to the following formula:

Extraction yield (mg of carotenoid per 100 g dry sample)

$$= \left(\frac{C_{calibrationcurve} \times 10 \times \Delta m_1}{m \times \Delta m_2}\right) \times 100 \Big/ 1000 \quad \text{(Equation 4)},$$

where $C_{calibration\ curve}$ (μg of carotenoid $mL^{-1}$): the concentration of total carotenoids from calibration curve, 10 (mL): dilution volume of measured extracts, m (g): the weight of the initial dried sample, $\Delta m_1$ (g): the weight of dry residue after rotary evaporation and $\Delta m_2$ (g): the weight of dry residue after $N_2$ evaporation.

### 5.7 Experimental design (DOE) models

The protocol for the optimization of high energy extraction methods contains three sequential steps, a) the preliminary experiments, b) the screening of the selected independent variables via a two-level design in order to direct extraction process to a more precise and limited experimental region of process parameters and c) the detection of optimal conditions for the carotenoid

extraction by applying a three-level response surface methodology (RSM) design [184].

A two-level full factorial design, $2^3$ and a symmetrical 16-run three-level Box-Behnken design (BBD) were selected for screening and RSM purposes. DOE extraction factors were a) extraction time, $X_1$ (min) b) US/MW power, $X_2$ (W) c) solvent/material ratio, $X_3$ (mL $g^{-1}$). The impact of the above extraction factors on carotenoids content was evaluated through the assessment of factors' main effects and interactions [138].

The transformation of the variables' real values ($X_1$, $X_2$, $X_3$) to encoded dimensionless values ($x_1$, $x_2$, $x_3$) is mandatory since the codification facilitates the normalization and comparison of the variables, which have different units and orders of magnitude [138]. The relationship between the coded and the real x values is expressed in the following equation (Eq. 5):

$$x_i=(X_i-X_0)/\Delta X \quad \text{(Equation 5)}$$

where $x_i$: the coded value for the independent variable, $X_i$: the real value for the independent variable, $X_0$: the real value for the independent variable at the center point, $\Delta X$: the step change value of the independent variable.

The experimental data of the extraction techniques were fitted to a first-order (for $2^3$ full factorial design) (Eq. 6) and a second-order polynomial model (for BBD design) (Eq. 7) that correlates the response with the independent variables according to the following functions:

$$y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{2} \sum_{j=1+1}^{3} A_{ij} X_i X_j \quad \text{(Equation 6)}$$

$$y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 \sum_{i=1}^{2} \sum_{j=1+1}^{3} A_{ij} X_i X_j \quad \text{(Equation 7)}$$

where y: extraction yield (dependent variable), $A_0$: constant term of the equation, $A_i$, $A_{ii}$ and $A_{ij}$: estimated coefficients by Box–Behnken model, $X_i^2$, $X_i$ and $X_j$: the levels of independent variables that represent the quadratic, linear and interaction effects of these variables on the response, respectively.

The experimental results of $2^3$ and BBD models were analyzed with Statistica software package (Version 10, StatSoft, Inc., USA) and the required calculations were done at 95% confidence level ($p$-values ≤ 0.05). Statistica package was also used to provide all graphs of the present work.

## 5.8 Identification and quantitation of carotenoids by liquid chromatography-photodiode array-atmospheric pressure chemical ionization mass spectrometry (APCI(+) LC-PDA-MS/MS)

Liquid chromatography (LC) instrumentation was combined of a) a quaternary pump b) an autosampler with a column oven set at 10 °C (Accela, Thermo Scientific, USA) c) an Acclaim C30 reversed-phase column (3 μm particle size, 150×2.1 mm i.d) thermostatted at 20 °C and d) a guard column. The injection volume was set at 5 μL and mobile phase flow rate at 350 μL/min. C30 column was selected instead of C18 columns due to the higher resolution of geometric isomers, the better selectivity and spectra quality, the shorter chromatographic time required and the more efficient separation of non-polar carotenoids (lycopene, b-carotene, etc) [47].

Mobile phase solvents were (A) acetonitrile (ACN) (B) methanol (MeOH) and (C) methyl-*tert*-butyl ether (MTBE). The eluting gradient program was the following: 0-5 minutes (30% A, 70% B), 5.1-13 minutes (22.9% A, 65.8% B and 11.3% C), 13.1-14 minutes (5% A, 75% B and 20% C), 14-14.1 minutes (30% A, 70% B) and 14.10-20 minutes (30% A, 70% B). MeOH-MTBE 50:50 v/v was samples and standard solutions solvent. No modifiers were added to the mobile phase as acetic and formic acid reduced m/z intensities. Since the above mobile phase is also applied for carotenoids analysis in lipid fractions and matrices, ammonium acetate was not the most ideal modifier as it enhances the ionization of various polar lipids interfering that way in carotenoids analysis [185].

PDA detector was set at 424, 445 and 455 nm. Ion trap mode of LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, USA) was used for MS identification. MS/MS measurements were performed on positive mode using atmospheric pressure chemical ionization (APCI) source at mass scan width 150-650 m/z. Although electrospray ionization (ESI) has been used for carotenoids fragmentation, APCI is considered the most appropriate ionization technique for less polar compounds, especially for carotenes [47].

Source parameters were optimized by applying a Plackett-Burman design (Supplementary data, Table S1). Screening of the APCI parameters revealed

that carotenoids' intensity was higher at high levels of S-LENS RF amplitude, vaporizer and capillary temperature and low levels of sheath, auxiliary and sweep gas flow. Normal probability plots (Supplementary data, Figure S2) identified S-LENS RF amplitude and sweep gas flow rate as the parameters affecting most carotenoids intensity.

A further optimization led to the final optimal values, which were determined as: S-LENS RF amplitude level=63%, vaporizer temperature=400 °C, sheath gas flow rate=25 a.u, auxiliary gas flow rate=5 a.u, sweep gas flow rate=0 a.u, discharge current=4 μA and capillary temperature=300 °C. ISO mass width was equal to 2.0. Collision energies for internal standard, b-carotene and zeaxanthin were set at 40 eV, while for lutein was adjusted at 35 eV.

Polyester filters (15 mm diameter, 0.45 μm pore size, Macherey-Nagel, Duren, Germany) were utilized for samples filtration. LC-MS/MS data were processed with Xcalibur software (Thermo Scientific, USA) and Plackett-Burman design was carried out using Statistica package (Version 12, Stat Soft, Inc., USA).

### 5.9 Nuclear magnetic resonance (NMR) metabolomic study

A Varian-600 MHz NMR spectrometer (Varian, USA) was used for acquiring NMR spectra. All spectra were obtained at ambient temperature (25 ºC) with a triple resonance {HCN} probe.

#### 5.9.1 NMR measurements

One dimension-Nuclear Overhauser effect spectroscopy (1D-NOE) pulse sequence was applied for 1H-NMR spectra. Spectra acquisition was performed at 128 transients collected with 128 K data points, spectral width of 7163.9 Hz, relaxation delay=1 s, acquisition time 4.454 s and mixing time=200 ms. Receiver gain was constant during all acquisitions.

Two dimension (2D) experiments gCOSY, gHMBCad, gHSQCad, recorded at 25 °C, enabled the identification of extracts metabolites. Analysis of 2D spectra was carried out using MestReNova v.10.1 software. Metabolites elucidation was facilitated by 2D NMR spectra plus reported data and cross-referenced with web-server metabolite database Metaboneer, an in-house fully automated metabolite identification platform [186].

### 5.9.2 Sample preparation for NMR measurements

Twenty milligrams (20 mg) of extracts dry residue were dissolved in 550 μL of $d_4$-methanol (apricot pulp) (Sigma-Aldrich, USA) or d-chloroform (Sigma-Aldrich, USA) (egg yolk and shrimps) and then 50 μL of $d_4$-TSP 5 mM diluted in methanol (internal standard) were added in a coaxial tube. Samples were transferred to 5 mm NMR tubes.

### 5.9.3 Data reduction and spectral alignment

All spectra were processed by MestReNova v.10.1 software for phasing, baseline correction, removal of methanol peak, binning into spectral buckets of 0.001 ppm and normalization to the reference compound standardized area. All spectra were converted to ASCII format and then imported into MATLAB (R2006a, Mathworks, Inc. 2006, Natick, MA, USA), where they were aligned using Correlation Optimized Warping (COW) method.

### 5.9.4 Multivariate data analysis

The SIMCA-P version 14.0 (Umetrics, Umeå, Sweden) was used for statistical processing of NMR data from apricot pulp analysis. The first step was the acquisition of a general overview and the visualization of the trends and outliers among apricot extracts by applying the exploratory principal component analysis (PCA).

A PCA model assesses the systematic variation in a data matrix by a low dimensional model plane. Further analysis of NMR data set occurred with supervised OPLS-DA models in order to estimate the between-class and within-class variation. All model were derived at 95% confidence level after mean-centered with Pareto scaling, which includes in the model low/medium intensity metabolites only if they display systematic variation [171].

The extraction variables and conditions that single out for their class discriminating power were revealed from loading plots. According to the definition of Umetrics (umetrics.com) [187] ‘’geometrically, the principal component loadings express the orientation of the model plane in the K-dimensional variable space (Fig. 5.3). The direction of PC1 in relation to the original variables is given by the cosine of the angles $\alpha_1$, $\alpha_2$, and $\alpha_3$. These

values indicate how the original variables $x_1$, $x_2$, and $x_3$ "load" into or contribute to PC1. Hence, they are called loadings. Of course, a second set of loading coefficients expresses the direction of PC2 in relation to the original variables. Hence, with two PCs and three original variables, six loading values (cosine of angles) are needed to specify how the model plane is positioned in the K-space''.

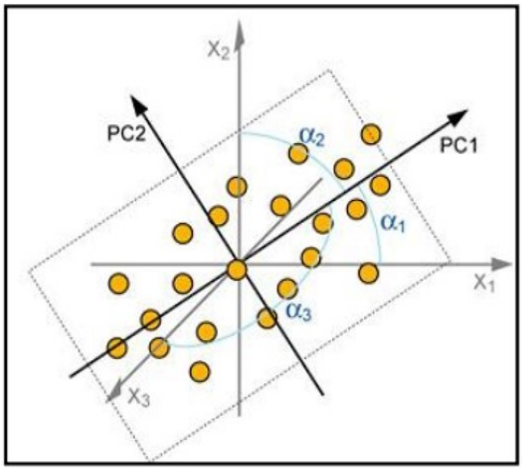



**Figure 5.3. Loading plot.**

Loading plots are used for the interpretation of PCA models. The representation of the experimental observations samples in one of the four quadrants of the PCA model defines also the position of the metabolites in the corresponding loading plot. The location of an experimental observation in the PCA quadrants is shown in Figure 5.4.

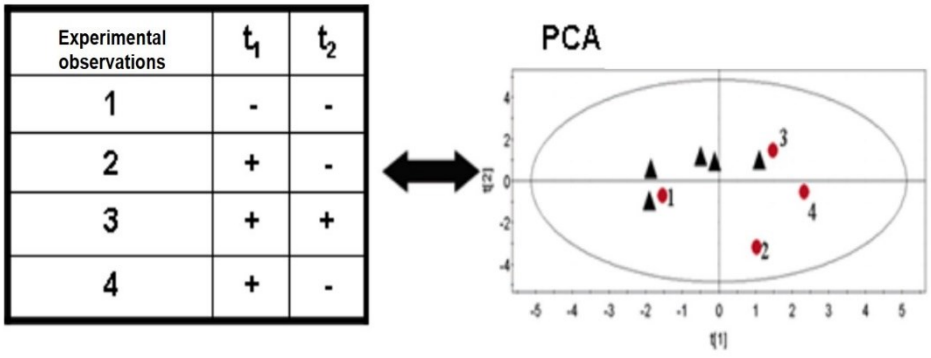



**Figure 5.4 Location of experimental observations in the quadrants of a PCA model.**

Models goodness-of-fit and predictive ability was evaluated by $R^2$ ($0 \leq R^2 \leq 1$) and $Q^2$ ($0 \leq Q^2 \leq 1$) values, respectively. $R^2$ refers to the data variance interpreted by the model, while cumulative $Q^2$ describes the variance of the data which are predictable by the model. In OPLS-DA models, statistical importance of $R^2$ and $Q^2$ is evaluated through a response permutation testing (999 permutations employed in our study) and a receiver operating characteristic (ROC) curve [171].

In permutation test plot, a model is valid when the intercept of $Q^2$ regression line is lower than zero and intercept of $R^2$ regression line crucially lower than that of the original [188]. A model is also considered significant when ROC values are ≥0.75. S-line plots highlighted the metabolites that contributed to DOE extracts discrimination.

# CHAPTER 6
# ULTRASOUND-ASSISTED (UAE) AND MICROWAVE-ASSISTED EXTRACTION (MAE) EXPERIMENTS

## 6.1 Preliminary experiments

In preliminary experiments, (a) extraction solvent, (b) extraction temperature, (c) sonication pulse sequence and (d) microwave ramping time were examined and their selected values were kept constant throughout all DOE experiments.

### 6.1.1 Extraction solvent

The most critical step in every extraction process is the selection of suitable extraction solvent based on its properties, on the nature of the examined matrix, on the solubility of extracted analytes and on the mechanism of the extraction procedure.

In general, UAE solvent efficiency depends on physical properties, like surface tension, vapor pressure and viscosity. In most cases, solvents with low viscosity and vapor pressure facilitate acoustic cavitation phenomenon and therefore extraction efficiency. Nonetheless, in some cases, these solvents could enhance localized pressure and temperature, which could result to loss of compound bioactivity and destruction of its structure. Hence, when an analyte shows similar polarity with an extractant of higher vapor pressure, UAE using this extraction solvent could take place in order to prevent compound degradation [142].

On the other hand, the determinant property of an adequate microwave (MW) solvent is the value of its dielectric constant and consequently, its transparency to MWs. Since MAE mechanism is a result of dipole rotation and ionic conductivity, solvents with high dielectric constant (usually more polar solvents) absorb efficiently MWs and become more suitable for MAE A transparent MW solvent with small dielectric constant cannot absorb MW energy and increase extraction temperature. Therefore, lower extraction yields are acquired. Nevertheless, totally or relatively transparent MW solvents (*n*-hexane and chloroform), and their mixtures with highly MW absorbing solvents (acetone and

methanol) could result to efficient extraction yields of sensitive labile compounds (phenolics, carotenoids, etc.) [88].

Extraction time, extraction power, solvent/material ratio and extraction temperature were set at 15 min with sonication pulse sequence at 15 s ON and 5 s OFF, 485.7 W, 20 mL $g^{-1}$ and 35 °C, for UAE, and at 10 min, 70 W, 20 mL $g^{-1}$ and at 50-65 ºC (according to the different boiling point of each solvent) for MAE, respectively throughout all solvent selection experiments in all three substrates. All results were delivered by APCI(+) LC-MS/MS analysis.

**i) Apricot byproducts**

Based on the lipophilic nature of carotenoids and the related literature [189], the different extraction systems that were investigated are shown in Figure 6.1. Methanol, ethanol, chloroform, acetone, n-hexane and their mixtures were the solvents tested. Alcohols and their mixtures provided the higher extraction yields in both techniques in accordance to the results exhibited in Supplementary data, Table S2a.

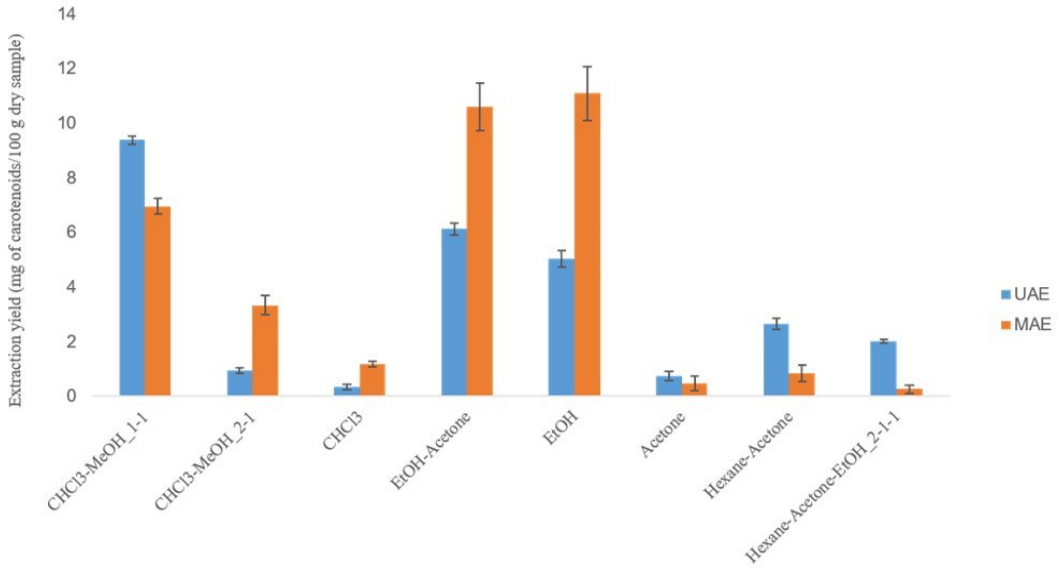



**Figure 6.1. Selecting optimal UAE and MAE solvent system for apricots pulp.**

All examined solvent exhibit similar values of surface tension, but methanol and ethanol have significantly lower vapor pressure compared to the other extractants. This property renders these two alcohols as efficient solvents for UAE of apricots carotenoids. Between the two alcohols, methanol has lower viscosity than ethanol and therefore its combination with a solvent of also low

viscosity (chloroform) provided the higher extraction yields [182]. Thus a **1:1 mixture of methanol:chloroform** was the selected solvent system for apricots UAE.

As stated above, normally polar solvents are more compatible with MAE. Among the solvent systems under study, **ethanol** is the most polar system and probably this is the reason why ethanol emerge as the best extraction solvent of carotenoids from apricot byproducts [190].

**ii) Egg yolk**

In egg yolk extraction, solvents that fit the needs of xanthophylls analysis were chosen for US and MW carotenoid extraction since the main carotenoids of egg yolk are lutein and zeaxanthin. Additionally, the different nature of egg yolk matrix (lipid substrate) compared to the apricot byproducts was taken into consideration in the selection of studied extraction solvents [189].

Chloroform, n-hexane:acetone 1:1 (v/v), chloroform:methanol 1:1 (v/v), ethanol and chloroform:methanol 2:1 (v/v) mixtures were tested (Fig. 6.2) in order to choose the most adequate solvent for each extraction technique. Chloroform, n-hexane:acetone 1:1 (v/v) and chloroform:methanol 2:1 (v/v) mixtures presented the highest values of extraction yields. As it is shown in Supplementary data, Table S2b, MAE egg yolk carotenoid yield is almost similar ($p$-value > 0.05) in all cases since all three solvent systems are appropriate for extracting lipophilic compounds, in accordance with Saini & Keum (2018) findings [189]. **Chloroform** was MAE solvent of choice as it achieved high carotenoid yields and is capable to extract not only the free forms of carotenoids but also carotenoids distributed in the phospholipid fraction [191].

On the contrary, UAE extraction yield seems to depend crucially on the physical properties of solvent system (Supplementary data, Table S2b). Chloroform, MAE optimal solvent, provided the lower extraction yield in UAE compared to the two other solvent systems used. The high vapor pressure of chloroform is probably responsible for the reduced recovery of macular carotenoids when US were used. On the other hand, as it is shown in Supplementary data, Table S2b, extraction systems of low vapor pressure solvents (n-hexane or methanol)

combined with higher vapor pressure solvents (acetone or chloroform), which presents molecular affinity to the target analytes, achieves substantially increased carotenoid yields. Thus, a 1:1 n-hexane:acetone and a 2:1 chloroform:methanol mixtures were used as UAE extraction solvent [192]. Among these two systems, a **1:1 mixture of n-hexane:acetone** provided higher UAE carotenoid yields according to Figure 6.2. .

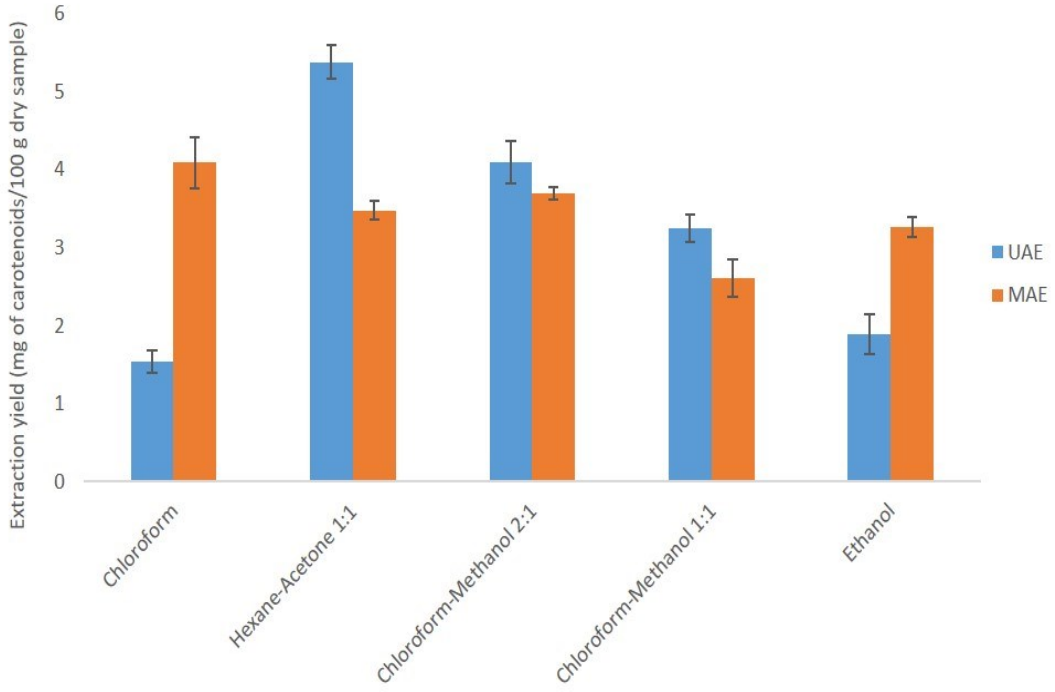



**Figure 6.2. Selecting optimal UAE and MAE solvent system for egg yolk.**

## iii) Shrimp head and body

For shrimps extraction, acetone, ethanol, a 1:1 (v/v) isopropanol (IPA):n-hexane mixture, a 2:1:1 (v/v) n-hexane:acetone:ethanol mixture and a 2:1:1 (v/v/v) petroleum ether:acetone:ethanol mixture were tested as extraction solvents (Figure 6.3). Petroleum ether and IPA are solvents commonly used in prior studies for the extraction of carotenoids, especially astaxanthin, from shrimps [193]. Shrimp body was used as the matrix for the optimization of extraction solvent as its content in carotenoids is lower than that of shrimp head. Thus an extraction system that achieves to extract efficiently carotenoids from shrimp body, would also present the same performance in shrimp head [54, 194].

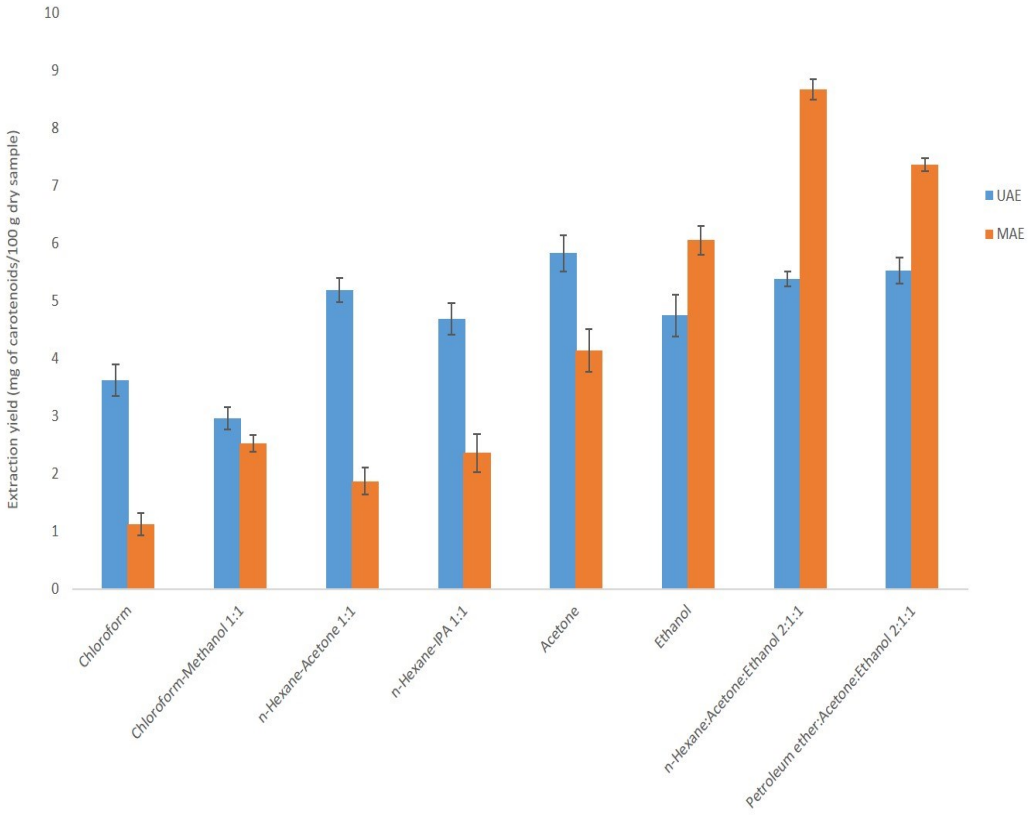



**Figure 6.3. Selecting optimal UAE and MAE solvent system for shrimps.**

In UAE, the higher extractability of carotenoids was achieved using acetone as solvent. As it is already known, the recovery of the extracted compounds is mainly, attributed to the acoustic cavitation phenomenon. This phenomenon is enhanced when solvents with low vapor pressures or solvents with low viscosity are used. Nevertheless, in that case, the extreme localized increase of pressure and temperature can cause degradation of the target compounds. Thus, solvents with higher vapor pressures which also present similar polarity with the target compounds are preferred [143]. Additionally, low viscosity solvents are diffused easier in the cells and facilitate acoustic cavitation [142]. **Acetone** is a 'generally recognized as safe' solvent with medium polarity, high vapor pressures and low viscosity and for these reasons was chosen as UAE solvent for the recovery of the lipid fraction that contains shrimp xanthophylls (Supplementary data, Table S2c).

In MAE, the heating efficiency of solvents that absorb MW, for example ethanol, is significant and in consequence, these solvents give high extraction yields. n-Hexane is a microwave transparent solvent and helps to the prevention of

degradation of carotenoids, which are thermolabile compounds. Acetone, although is not a highly polar solvent, has a high dissipation factor value, therefore an efficient heating occurs. A mixture of solvents with these physicochemical properties normally provides optimum extraction yields, as a crucial fraction of MW is absorbed by the matrix and the efficiency of the extraction is larger than that of solvent mixtures with high dissipation values, for example, water [88, 195, 196]. Hence, a **2:1:1 mixture of n-hexane:acetone:ethanol** was selected as the most suitable solvent for MAE of xanthophylls (Supplementary data, Table S2c).

### 6.1.2 Extraction temperature

Temperature is considered as an important parameter of the extraction process since it can affect positively or negatively the recovery of thermosensitive compounds. The raise of extraction temperature enhances the distribution of extracted analytes to the solvent due to the rupture of the matrix cells or granules, but on the other hand high temperatures could lead to the deterioration of thermosensitive compounds [191].

The extraction yield, in UAE, is a result of the combination of acoustic cavitation and thermal activities. Extraction temperature exhibits ambiguous effect during UAE process, since raised temperatures increase solvent vapor pressure. Higher vapor pressures diminish acoustic cavitation intensity and consequently UAE yields [145]. Thus, low temperatures are recommended for UAE in order to avoid increase of solvent's vapor pressure which limits the collapse of cavitation bubbles and, by extension, the appearance of sonochemical effects [8]. Hence, in the current project, UAE temperature was set, for all three substrates, at relatively low values (30–35 °C) by placing UAE flask in an ice bath during probe sonication procedure.

In open vessel MAE, the extraction temperature is interrelated with applied MW energy and extraction time. However, it never exceeds the boiling point of extraction solvent. The absorption of MW results to the increase of temperature and heating of the solvent that leads to the faster and complete rupture of the matrix cells and therefore, to higher extraction yields, as an accelerate diffusion of analytes from the matrix to the solvent takes place [88, 197]. Therefore, MAE

temperature was set at 55-65 ºC based on the solvent selected for each substrate and solvent's boiling point. According to published works, all forms of carotenoids are stable in these temperatures [198, 199].

### 6.1.3 UAE sonication pulse and MAE ramping time

According to the literature [145] and the preliminary results [146], a pulsed sonication, in UAE, is a more efficient and energy saving method compared to continuous sonication. As there was no significant difference on carotenoid yield at different pulse intervals, a pulse sequence of 15 s ON and 5 s OFF was applied.

Based on the results of preliminary experiments [146], ramping time was set to zero since longer ramping times in MAE, increase the total extraction time and result to lower yields of carotenoids.

## 6.2 DOE-based optimization of UAE and MAE

The next phase of optimization protocol, after preliminary experiments, is the experimental design (DOE) optimization of high energy extraction methods, which was performed in two steps: (a) the screening of the selected independent variables via a two-level design in order to direct extraction process to a more precise and limited experimental region of process parameters and (b) the detection of optimal conditions for the carotenoid extraction by applying a three-level response surface methodology (RSM) design [184].

The purpose of screening experiments was to diminish and define accurately factors' experimental range in which UAE and MAE perform better. In more details, screening design was applied in order to i) limit the wide values' range of the selected factors in a region where higher yields were achieved and ii) direct the imminent optimization model (response surface model) to this region. For achieving this aim, a $2^3$ full factorial design with an extreme upper (+1) and an extreme lower (−1) value of each variable was employed. The eight experimental runs were delivered in random order for evading potential spurious systematic errors [73].

Response surface methodology (RSM) models were applied in order to define the exact optimal values for extraction procedures. In this work, a symmetrical

three-level Box-Behnken design (BBD) was implemented. The complete experimental planning for BBD of UAE and MAE parameters consists of sixteen experimental combinations in random order to avoid possible artificial systematic effects, with four replicates at the center point. The repetitions at the center point are necessary for the estimation of pure error associated with them, which is an index of the repeatability and robustness of each model [200].

The extraction factors under DOE-optimization, in all cases, were: (a) extraction time (min, $X_1$), (b) MW or US power (W, $X_2$) and (c) solvent/material ratio (mL $g^{-1}$, $X_3$). By implementing this design, variables main effects and their interactions' effects on the response (i.e. carotenoid content as mg of carotenoids per 100 g dry sample) was estimated. As each variable refers to different physical units and has a distinct range of experimental values, the importance of these parameters can be estimated easier when their levels are normalized and coded in comparable values ($x_1$, $x_2$, $x_3$). Carotenoid content was estimated as a function of the principal carotenoid of each substrate using *vis*-spectrophotometry as it is a quick and straightforward method.

In general, goodness-of-fit of the models was assessed using the determination coefficient ($R^2$) and the determination coefficient adjusted for the degrees of freedom ($R^2_{adj}$).The first index indicates how well the produced models fit to the dataset and the second one determines which terms of the equation, proposed by the models, truly affect the response. A model where the values of the two coefficients are higher than 0.8 and their difference around 0.2 describes well the dataset.

### 6.2.1 Apricot byproducts

The effect of extraction parameters on carotenoids extraction yield was measured spectrophotometrically and expressed as b-carotene content. All experimental runs were performed using a 1:1 v/v mixture of methanol-chloroform, for UAE and ethanol, for MAE. The real and coded values of $2^3$ and BBD for UAE and MAE are presented in Table 6.1.

**Table 6.1. Normalized and real values of UAE/MAE experimental factors for 23 and BBD design for apricot byproducts.**

| *Coded values* | *-1* | *0* | *+1* |
|---|---|---|---|
| | | **$2^3$ design** | |
| ***UAE*** | | | |
| **Extraction time ($X_1$, min)** | 5 | | 35 |
| **US power ($X_2$, W)** | 375 | | 675 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 10 | | 35 |
| ***MAE*** | | | |
| **Extraction time ($X_1$, min)** | 5 | - | 30 |
| **MW power ($X_2$, W)** | 70 | - | 200 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 20 | - | 60 |
| | | **BBD** | |
| ***UAE*** | | | |
| **Extraction time ($X_1$, min)** | 10 | 20 | 30 |
| **US power ($X_2$, W)** | 577 | 622 | 675 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 25 | 30 | 35 |
| ***MAE*** | | | |
| **Extraction time ($X_1$, min)** | 5 | 10 | 20 |
| **MW power ($X_2$, W)** | 60 | 90 | 130 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 45 | 65 | 55 |

The experimental combinations, in coded values, and the extraction yields of $2^3$ design experiments, for apricot pulp, are shown in Supplementary data, Table S3.

Regarding apricot byproducts extraction, $2^3$ design could direct reliably the upcoming Box-Behnken model to the value range where higher carotenoid yields would be obtained since $R^2$=0.920, $R^2_{adj}$=0.814 for UAE and $R^2$=0.987, $R^2_{adj}$=0.954 for MAE. Based on *p*-values, special attention should be paid at the adjustment of solvent/material ratio ($x_3$) (*p*-values=0.042, for UAE, and *p*-

values=0.0081, for MAE) in both extraction techniques. According to two-dimensional (2D) contour plots UAE performed better at extraction times over 10 minutes when US power and solvent/material ratio were set at high values (≥600 W and ≥30 mL $g^{-1}$) (Fig. 6.1a-c). Based on contour plots of MAE parameters, the sequent BBD optimization model for apricot byproducts should be focused on extraction times from 5-20 minutes, MW power lower than 100 W and high solvent/material ratio (≥50 mL $g^{-1}$) (Fig. 6.4d-f).

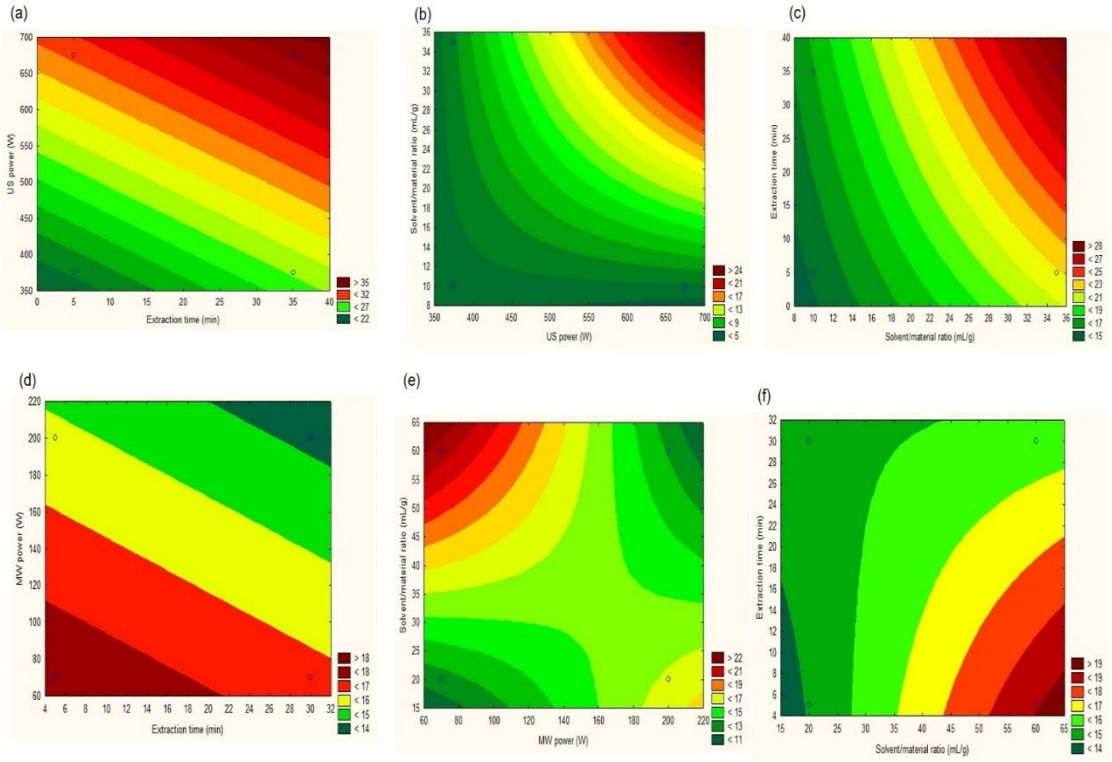



**Figure 6.4. Contour plots of $2^3$ design for UAE (a-c) and MAE (d-f) of apricot pulp.**

Box-Behnken optimization models, driven by the trends revealed in the prior screening designs, were applied for accomplishing the optimal extraction values of high energy techniques. The model-proposed experimental runs and carotenoid content for each run are illustrated in Supplementary data, Table S3.

The processing of dataset resulted in two (one for each extraction method) predictive second-order polynomial equations, which involve quadratic terms, linear terms and their interaction terms. Terms with high *p*-values (*p*-value≥0.05) were considered statistically insignificant and excluded from the equations. Thus, the final model equations, expressed in normalized values, (Eq. 8-9) consisted of the following terms:

$$\text{UAE yield (mg of carotenoids 100 g}^{-1}\text{ dry sample)}=8.706-1.80x_1+1.003x_1^2+0.80x_2^2+1.021x_3^2+0.5280x_1^2x_2+0.4674\ x_1x_2^2-1.761x_1x_3-1.819x_1^2x_3+2.023x_2x_3 \quad \text{(Equation 8)}$$

$$\text{MAE yield (mg of carotenoids 100 g}^{-1}\text{ dry sample)}= 15.91-0.3208x_1^2+2.767x_2-1.116x_2^2+1.372x_1x_2+1.554x_1x_2^2-2.332x_1x_3-3.784x_2x_3 \quad \text{(Equation 9)}$$

The significance of all equation terms is illustrated in Pareto charts (Fig. 6.5), where the factors and their interactions are listed from largest to smallest by the magnitude of their effect. The terms which are over the threshold (red line) of 95% confidence level (*p*-value≤0.05) are characterized as significant. Taking into consideration the *p*-value≤0.05 criterion presented in ANOVA table (Supplementary data, Table S4b), UAE was significantly affected by (i) the linear term of extraction time ($x_1$), (ii) the quadratic term of solvent/material ratio ($x_3^2$) and (iii) the interaction of solvent/material linear term with (a) extraction time quadratic term ($x_1^2x_3$) and (b) US power linear term ($x_2x_3$). UAE yields presented a directly proportional linear relationship to extraction time and a directly proportional exponential relationship to solvent/material ratio explained by the positive sign of $x_1$ and $x_3^2$ terms in Equation 8.

Furthermore, both (i) linear ($x_2$)and (ii) quadratic term ($x_2^2$) of MW power, (ii) the interaction of extraction time linear term and MW power quadratic term ($x_1x_2^2$) and (iv) the interaction of solvent/material linear term with (a) the linear terms of extraction time ($x_1x_3$) and (b) MW power ($x_2x_3$) played the most important role in MAE carotenoids yields (Supplementary data, Table S4b). As illustrated in Figure 6.5b, the linear term of MW power affected more the final result than the quadratic term. Thus, the positive sign of $x_2$ term in Equation 9 revealed the directly proportional dependence of MAE yields and MW power.

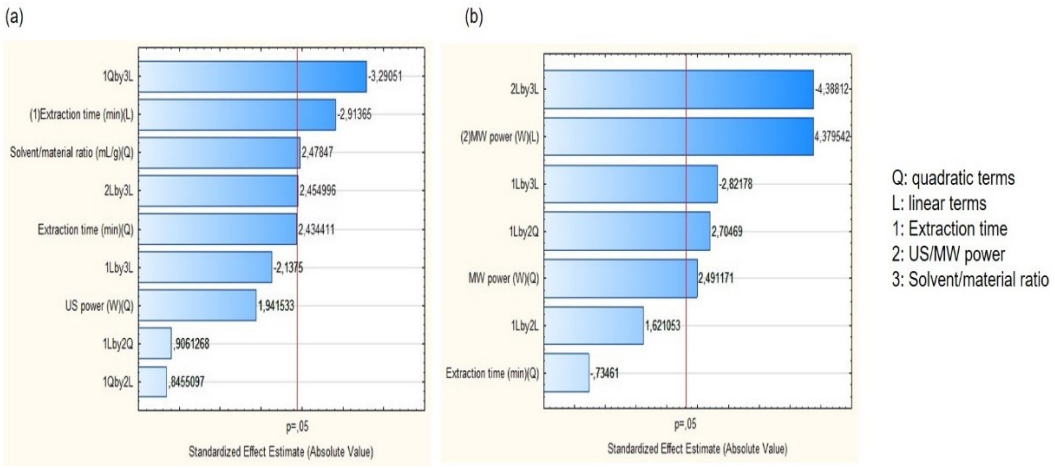



**Figure 6.5. Pareto charts for (a) UAE and (b) MAE of apricot byproducts.**

The BBD models were considered reliable according to $R^2$ and $R^2_{adj}$ values, which were relatively high and close to one another ($R^2$=0.886 and $R^2_{adj}$=0.714 for UAE and $R^2$=0.876 and $R^2_{adj}$=0.767 for MAE). Moreover, only a percentage of around 10% of the total variations is not interpreted by the produced models. The good fitness of the BBD models in the experimental data was also established by the *p*-values corresponding to the total model and not on each term, which were ≥0.05 (*p*-value UAE=0.941, *p*-value MAE=0.979) confirming that there was no models' lack-of-fit (Supplementary data, Table S4b). In addition, the robustness of our models was evaluated through the standard deviations (UAE stdev=2.2, MAE stdev=2.6) of the four repetition at the center points (0,0,0) (Supplementary data, Table S3).

Three-dimensional (3D) response surface methodology (RSM) plots (Fig. 6.6a-f) were generated for the evaluation of the DOE-optimized extraction factors on carotenoids yield. RSM plots depict the combinatorial effect of two of the investigated extraction factors on the carotenoid content of apricot byproducts, while the third parameter is kept constant at the medium value level (0).

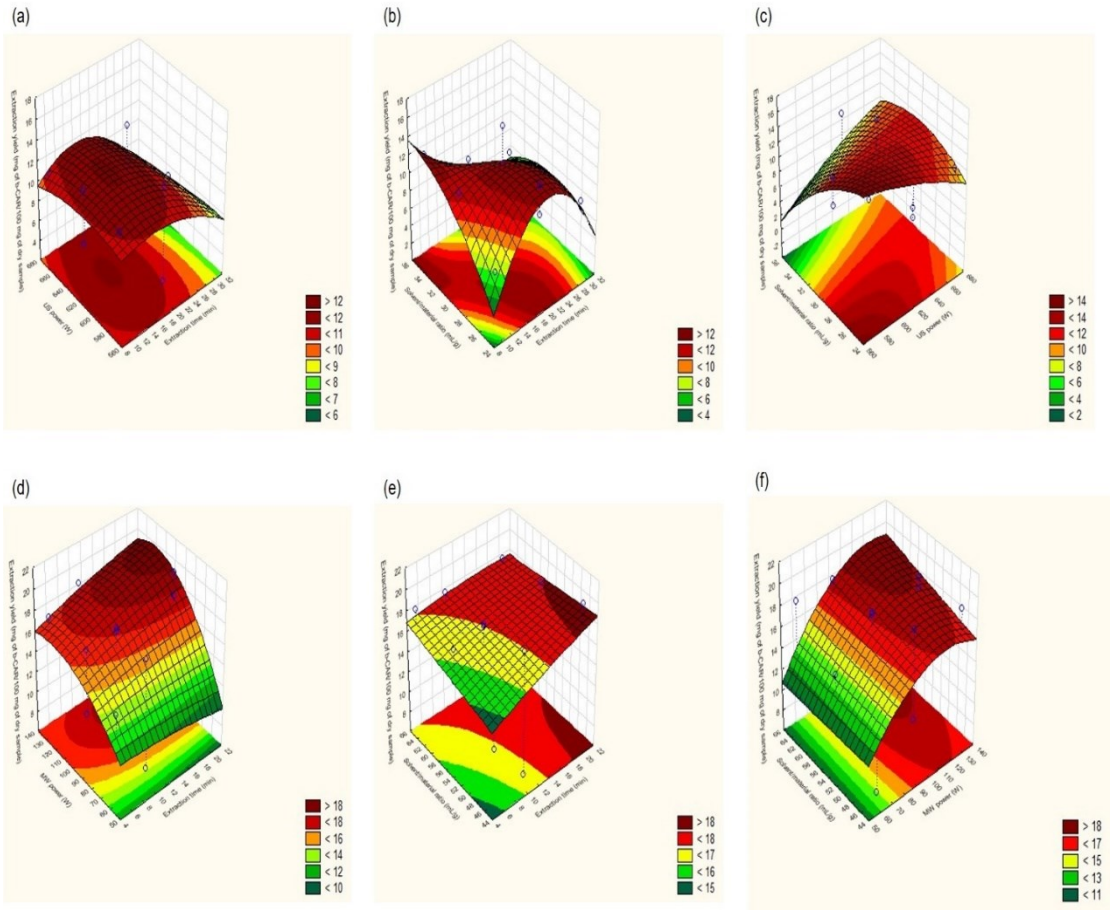



**Figure 6.6. RSM plots for UAE (a-c) and MAE (d-f) of apricot pulp.**

## i) Effect of extraction time

As exhibited in Figures 6.6a and 6.6b, UAE was favored at extraction times between 10-20 minutes. A closer look at these figures showed that at even 10 minutes extraction time, high extraction could be achieved when US power and solvent/material ratio took values of 600-620 W and 30-35 mL $g^{-1}$. Studies at carotenoids UAE from plant tissues indicated that carotenoids may be degraded at prolonged extraction time (over 15 minutes). Thus, a 10 to 20-minute period seems ideal for carotenoids recovery from agro-byproducts [201].

Compared to UAE, MAE is a more complex process due to the interrelation of extraction time, temperature and MW power. Chuyen et al (2018) [202] examined the co-dependance of these three parameters and showed that extended extraction times over 15 minutes resulted in high carotenoids yields when MW power was adjusted at 100-140 W [202]. This was also confirmed in

our work (Fig. 6.6d). In addition, higher extraction time and higher solvent/material ratio provided higher carotenoids content (Fig. 6.6e).

**ii) Effect of US/MW power**

In general, the positive impact of sonochemical effects is more pronounced at increased US power which improve cell walls breakage and solvent penetration. Recent researches asserted that US power over 250 W affect negatively the extraction efficiency but US exposure lasted for long periods (40-100 minutes) [8]. However, high US power (580-620 W) applied for short periods (8-15 minutes) could recover high concentrations of carotenoids (Fig. 6.6a and 6.6c).

An increasing trend on extraction yields was observed (Fig. 6.6d and 6.6f) at 100-140 W of MW power. At this power range, the increase of extraction temperature was slower and steady throughout MAE process. Thus, the release of target compounds from substrate tissues was more efficient due to more gradual solvent heating. Higher MW power may i) deteriorate the extracted labile molecules or ii) cause solvent losses from extreme solvent heating and lead to reduced extraction rates. On the other hand, low irradiation values do not succeed complete cell disruption and therefore extraction yields are usually lower [202].

**iii) Effect of solvent/material ratio**

According to UAE Pareto chart (Fig. 6.5a) solvent/material ratio was viewed as the most critical extraction factor. As showcased in Fig. 6.6b and 6.6c, solvent volumes between 25-35 mL seems to be enough to diffuse and dissolve adequately the extracted compounds. Addition of extra volume did not maximize carotenoids migration to the solvent due to the increase of the diffusion distance from the extractant medium and the examined matrix [203].

MAE regression equation (Eq. 9) indicated as quite significant terms the interaction of solvent/material ratio with MW power and extraction time (Fig. 6.5b). When extraction time varies from 15-20 minutes and MW power from 100-120 W, solvent/material ratio should be adjust at 44-56 mL for obtaining maximum extraction yields (Fig. 6.6e and 6.6f). Larger solvent volumes demanded longer periods (≥20 minutes) of MW irradiation at the mentioned

values in order to achieve a uniform solvent heating and thus an efficient carotenoids recovery. Nevertheless, extended extraction times of MW radiation could promote the degradation of thermosensitive compounds, like b-carotene or zeaxanthin [204].

**iv) Optimal extraction conditions**

The third and final step of DOE optimization strategies refers to the conduction of experiments around the value regions where the response is maximized according to the equations produced by BBD (Eq. 8-9). Three experimental combinations were proposed and performed as optimal (Supplementary data, Table S5). The lack of significant difference (Student's t-test) between the predicted and experimental values proved the reliability of DOE-optimized extractions. The predictive capability of the established models is presented in the plots of observed versus predicted values (Supplementary data, Figure S3). If a model predicts reliably all the points, which represent the experimental runs should lie on the diagonal line or should be scattered evenly on the above and below the diagonal line.

The **optimal values** of UAE and MAE parameters for carotenoids recovery from apricot byproducts are presented in Table 6.2.

**Table 6.2. Optimal values of UAE and MAE parameters for carotenoids recovery from apricot byproducts.**

| Extraction parameters | Optimal values | |
|---|---|---|
| | **UAE** | **MAE** |
| **Extraction solvent (v/v)** | Methanol:chloroform 1:1 | Ethanol |
| **Extraction time (min)** | 10 | 20 |
| **US/MW power (W)** | 600 | 120 |
| **Solvent/material ratio (mL $g^{-1}$)** | 35 | 45 |
| **US pulse sequence (s)/MW ramping time (min)** | 15 ON 5 OFF | 0 |
| **Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev), n=3[a]** | 11.12(±0.34) | 19.28(±0.27) |

[a*]n: number of sample replicates measured under repeatability conditions

### 6.2.2 Egg yolk

In egg yolk extraction, the effect of extraction parameters on carotenoids extraction yield was measured spectrophotometrically and expressed as lutein content. All experimental runs were performed using a 1:1 v/v mixture of n-hexane-acetone, for UAE and chloroform, for MAE. The real and coded values of $2^3$ and BBD for UAE and MAE are presented in Table 6.3.

**Table 6.3. Normalized and real values of UAE/MAE experimental factors for 23 and BBD design for egg yolk.**

| ***Coded values*** | ***-1*** | ***0*** | ***+1*** |
|---|---|---|---|
| | | **$2^3$ design** | |
| ***UAE*** | | | |
| **Extraction time ($X_1$, min)** | 5 | | 35 |
| **US power ($X_2$, W)** | 375 | | 675 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 10 | | 35 |
| ***MAE*** | | | |
| **Extraction time ($X_1$, min)** | 5 | - | 20 |
| **MW power ($X_2$, W)** | 70 | - | 170 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 30 | - | 60 |
| | | **BBD** | |
| ***UAE*** | | | |
| **Extraction time ($X_1$, min)** | 20 | 25 | 30 |
| **US power ($X_2$, W)** | 550 | 600 | 650 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 25 | 30 | 35 |
| ***MAE*** | | | |
| **Extraction time ($X_1$, min)** | 10 | 20 | 30 |
| **MW power ($X_2$, W)** | 160 | 180 | 200 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 20 | 30 | 40 |

The experimental combinations, in coded values, and the extraction yields of $2^3$ design and BBD experiments, for egg yolk, are shown in Supplementary data, Table S6.

The applied $2^3$ design showed a good fit, as both the determination coefficient ($R^2$) and the determination coefficient adjusted for the degrees of freedom ($R^2_{adj}$) were close and relatively high ($R^2$=0.872 and $R^2_{adj}$=0.701 in UAE and in MAE $R^2$=0.998 and $R^2_{adj}$=0.980). Hence, the applied $2^3$ designs indicated successfully the proper experimental settings of extraction factors in the BBD that comes next.

ANOVA (Supplementary data, Table S7a) revealed that among all parameters, more attention should be paid in UAE solvent/material ratio and MAE extraction time, as these factors seem to have the most prominent effects (p-values≤0.05) in extraction yields. Screening $2^3$ models indicated the optimal value range in order to construct BBD optimization model and maximize the response. Results of $2^3$ design can be depicted with the 2D contour plots (Fig. 6.7a-f). According to Figures. 2a-2c UAE will be optimized between 20 and 30 min, 550–650 W and 25–35 mL $g^{-1}$.

On the same ground Figures 6.7d-f, indicated that MAE will provide higher extraction yields when extraction time, MW power and solvent/material ratio vary between 10 and 30 min, 160–200 W and 20–40 mL $g^{-1}$, respectively. At values lower or higher of these ranges, extraction yields decrease either due to inefficient extraction or carotenoids losses.

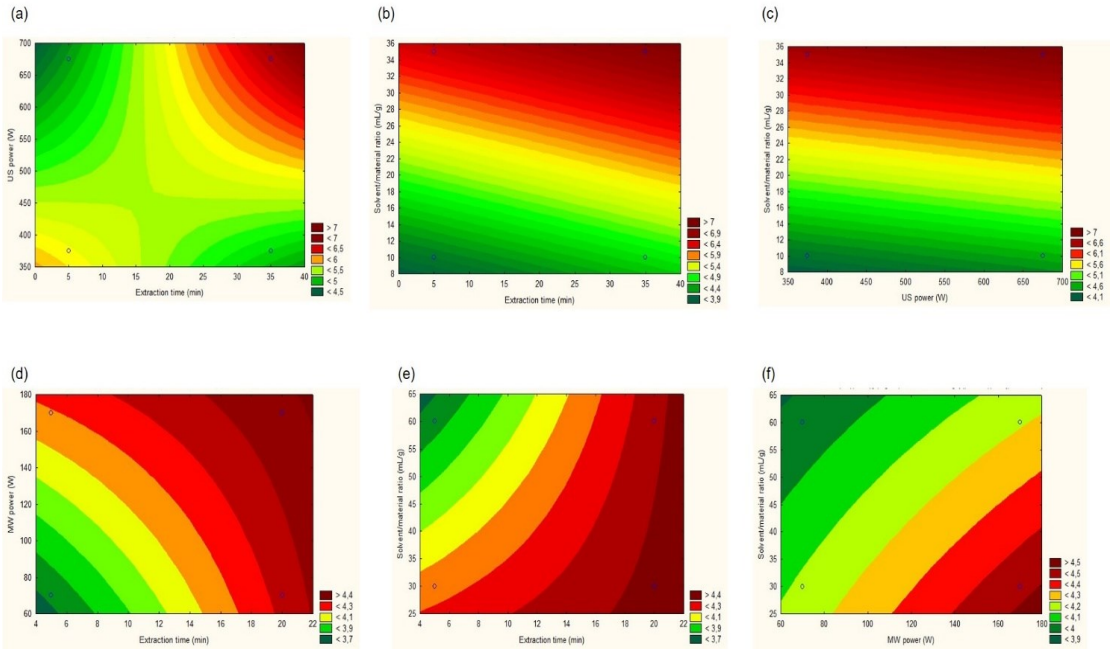



**Figure 6.7. Contour plots of $2^3$ design for UAE (a-c) and MAE (d-f) of egg yolk.**

BBD models of egg yolk extraction showed good fit when insignificant terms (p-value≥0.05) were excluded from the initial models. The final second-order polynomial equations (Eq. 10–11) are described below in terms of coded values:

$$\text{UAE yield (mg of carotenoids 100 g}^{-1}\text{ dry sample)}=6.7926-0.5189x_1^2+0.2969x_2+0.4137x_3-0.3258x_3^2-0.6127x_1x_2^2+0.4974x_1x_2-0.1910x_1^2x_2+0.1927x_1x_3-0.1365x_1^2x_3 \quad \text{(Equation 10)}$$

$$\text{MAE yield (mg of carotenoids 100 g}^{-1}\text{ dry sample)}=4.1075+0.2450x_1-0.2843x_1^2+0.0187x_2+0.2331x_2^2+0.3041x_3-0.0394x_3^2-0.0900x_1x_2^2+0.3550x_1x_3-0.1962x_1^2x_3 \quad \text{(Equation 11)}$$

According to p-values (≤0.05) from ANOVA table (Supplementary data, Table S7b) UAE (a) quadratic term ($x_1^2$) along with (b) the interaction of extraction time's linear term and US power's quadratic term ($x_1x_2^2$), have important influence on xanthophylls yield. Quadratic term of extraction time ($x_1^2$) affects crucially MAE extraction yield (Supplementary data, Table S7b). The importance of each extraction parameter is demonstrated in Pareto's charts (Fig. 6.8a-b).

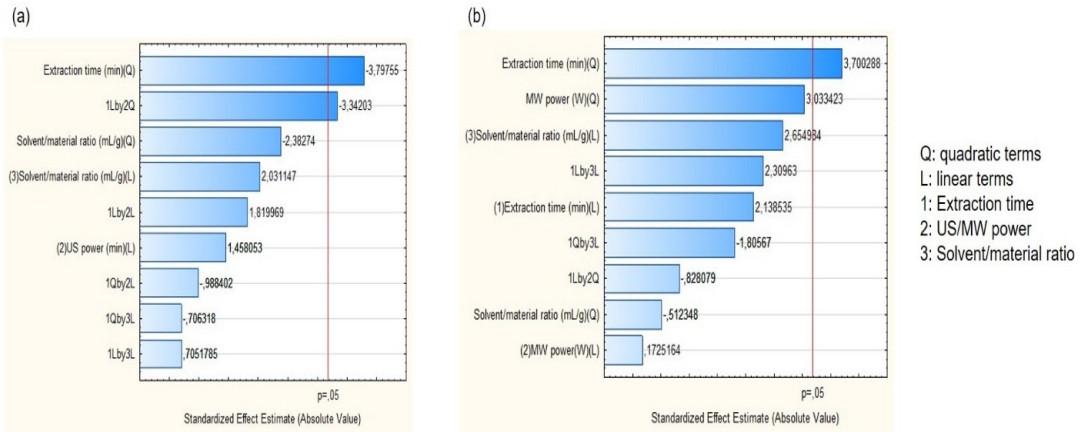



**Figure 6.8. Pareto charts for (a) UAE and (b) MAE of egg yolk.**

Adequacy of BBD models was confirmed by the values of $R^2$ and $R^2_{adj}$ ($R^2$=0.932 and $R^2_{adj}$=0.830 in UAE and $R^2$=0.924 and $R^2_{adj}$=0.811 in MAE) which are both high and close. Based on $R^2$ values only 0.068% of UAE and 0.076% of MAE total variations were not described by our BBD models. High $R^2_{adj}$ values in both extraction methods affirmed the significance of the models. Lack of fit p-values are higher than the significance level of 0.05 (UAE p-value=0.996 and MAE p-value=0.941), therefore our models fit excellent to the experimental data (Supplementary data, Table S7b). Furthermore, repeatability and reliability of BBD models were confirmed by low standard deviation values of the four replicates at central points (0,0,0) (Standard deviation UAE=0.55, Standard deviation MAE=0.31).

Main and interactive effects of extraction factors are depicted by using RSM 3D plots (Fig. 6.9a-f).

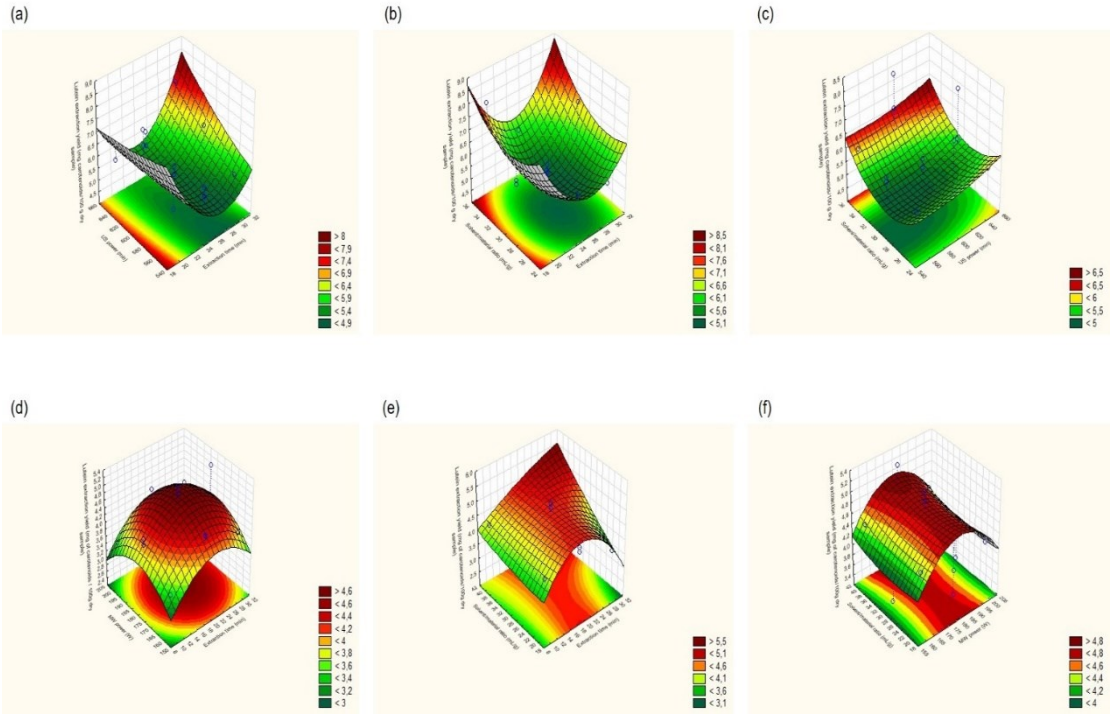



**Figure 6.9. RSM plots for UAE (a-c) and MAE (d-f) of egg yolk.**

## i) Effect of extraction time

UAE extraction yield seemed to increase at the upper or lower extreme point (≤20 min or ≥30 min) of extraction time experimental range (Fig. 6.9a and b). Taking into consideration the stability of carotenoids as well as the protection of US probe equipment from intense and long-term functioning, shorter extraction times were chosen for UAE.

On the other hand, Fig. 6.9d revealed that MAE extraction yield reached a local optimum from 20 to 25 min when MW power was set between 170 and 190 W. This conclusion, regarding optimal extraction time, is illustrated in Figure 6.6e where high carotenoids yields were achieved at extraction duration around 20 min when solvent/material ratio is over 35 mL $g^{-1}$. In time periods of 20 to 25 min extraction solvent could be heated adequately in order to result to a full recovery of egg yolk carotenoids without causing xanthophylls' deterioration [146].

## ii) Effect of US/MW power

Compared to MAE instrumentation, UAE probe permitted the irradiation of extraction mixture with higher power values (for example 50% of US probe

power is equivalent to 375 W). Thus, UAE procedure was performed under higher power values than MAE. Based on the information provided by Fig. 6.9a and c, xanthophylls recovery is more quantitative at US values over 500 W. By analyzing the 3D plots of US power (Fig. 6.9a and c), carotenoids' recovery was optimized at US power between 550 and 660 W when extraction time was around 20 min and solvent/material ratio over 35 mL $g^{-1}$. In general, higher US power values promote carotenoids' diffusion in extraction solvent due to more extensive granule break of the substrates [145].

The second most significant MAE parameter after the extraction time is the MW power. As illustrated in Figure 6.9d and f, extraction yield was enhanced when MW power varied from 170 to 190 W. Increase of MW power amplifies dipole rotation which leads to solvent heating and improves the solubility of target compounds in solvent mixture [205]. Moreover, higher MW power levels, over 190 W (Fig. 6.9d and f) led to the reduction of extraction yield, which could be attributed to the increase of solvent temperature and the consequent degradation of carotenoids.

**iii) Effect of solvent/material ratio**

In UAE, the effect of solvent/material ratio was also more crucial at values higher than 30 mL $g^{-1}$ (Fig. 6.9b and c). Acoustic cavitation augments the contact surface of egg yolk and 1:1 n-hexane:acetone, which is our extraction solvent system, facilitated in that way solvent penetration into matrix granules. In addition, when higher solvent volumes are employed in extraction systems, mass transfer rate is accelerated due to the concentration difference developed between the liquid and the substrate [189]. Thus, extraction yield presented significantly greater values.

Figures 6.9e and f depict solvent/material ratio influence on MAE yield. According to them, solvent/material ratio near 40 mL $g^{-1}$ is recommended. According to literature data [206], high solvent/material ratio could decrease extraction efficiency, as the homogeneous heating of total solvent volume requires longer exposure time compared to a MAE run carried out with lower solvent/material ratio. Chloroform is a solvent with low dielectric constant and thus, its interaction with MWs is relatively poor. Therefore, in that case, higher

solvent/material ratio enhances the ability of chloroform to selectively extract and solubilize the lipid fraction of a lipid matrix as egg yolk [207].

**iv) Optimal extraction conditions**

The developed BBD models unveiled the optimal area of the experimental range for extraction processes. Relying on the direction indicated by 3D RSM plots, three runs around the optimal values area were carried out in order to define the exact optimal combination of extraction conditions in each case (Supplementary data, Table S8). Based on the plots of predicted versus experimental values BBD models predicted accurately the carotenoids extraction yield of egg yolk, with MAE model showing an excellent predictability (Supplementary data, Figure S4). The **optimal values** of UAE and MAE parameters for carotenoids recovery from egg yolk are presented in Table 6.4.

**Table 6.4. Optimal values of UAE and MAE parameters for carotenoids recovery from egg yolk.**

| Extraction parameters | Optimal values | |
|---|---|---|
| | **UAE** | **MAE** |
| **Extraction solvent (v/v)** | n-Hexane-Acetone 1:1 | Chloroform |
| **Extraction time (min)** | 19 | 22 |
| **US/MW power (W)** | 600 | 182 |
| **Solvent/material ratio (mL $g^{-1}$)** | 35 | 40 |
| **US pulse sequence (s)/MW ramping time (min)** | 15 ON 5 OFF | 0 |
| **Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev), n=3[a]** | 7.41(±0.34) | 4.88(±0.18) |

[a*]n: number of sample replicates measured under repeatability conditions

### 6.2.3 Shrimp head and body

In shrimp head and body, the effect of extraction parameters on carotenoids extraction yield was measured spectrophotometrically and expressed as astaxanthin content. High energy extraction methods were optimized using shrimp body and then applied to shrimp head which always contains higher concentrations of carotenoids [54, 194]. All experimental runs were performed

using acetone, for UAE and a 2:1:1 mixture of n-hexane:ethanol:acetone, for MAE. The real and coded values of $2^3$ and BBD for UAE and MAE are presented in Table 6.5.

The applied $2^3$ design exhibited an exceptional goodness-of-fit, as both the determination coefficient ($R^2$) and the determination coefficient adjusted for the degrees of freedom ($R^2_{adj}$) were close and high ($R^2$=0.983 and $R^2_{adj}$=0.940 in UAE and in MAE $R^2$=0.908 and $R^2_{adj}$=0.785). Therefore, the produced $2^3$ designs directed successfully the imminent BBD model to the experimental range of extraction factors where extraction yields tend to be maximized.

According to ANOVA (Supplementary data, Table S10a) the parameters that have more importance (p-values≤0.05) for the optimization of high energy extraction methods were extraction time, US power and MAE solvent/material ratio. The results of $2^3$ design are demonstrated in the 2D contour plots (Fig. 6.10a-f). According to Figures 6.10a-c UAE will be optimized in short extraction times (2-8 min), high US power (600–650 W) and low to medium solvent/material ratios (10–25 mL $g^{-1}$).

On the other hand, Figures. 6.10d-2f, showed that MAE will achieve higher extraction yields when extraction time, MW power and solvent/material ratio vary between 2-8 min, 20–60 W and 10-20 mL $g^{-1}$, respectively.

**Table 6.5. Normalized and real values of UAE/MAE experimental factors for 23 and BBD design for shrimp body.**

| *Coded values* | *-1* | *0* | *+1* |
|---|---|---|---|
| **$2^3$ design** | | | |
| ***UAE*** | | | |
| **Extraction time ($X_1$, min)** | 2 | | 20 |
| **US power ($X_2$, W)** | 375 | | 675 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 10 | | 35 |
| ***MAE*** | | | |
| **Extraction time ($X_1$, min)** | 3 | - | 15 |
| **MW power ($X_2$, W)** | 30 | - | 120 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 10 | - | 30 |
| **BBD** | | | |
| ***UAE*** | | | |
| **Extraction time ($X_1$, min)** | 5 | 10 | 15 |
| **US power ($X_2$, W)** | 550 | 600 | 650 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 10 | 20 | 30 |
| ***MAE*** | | | |
| **Extraction time ($X_1$, min)** | 5 | 7 | 9 |
| **MW power ($X_2$, W)** | 30 | 40 | 50 |
| **Solvent/material ratio ($X_3$, mL $g^{-1}$)** | 10 | 20 | 30 |

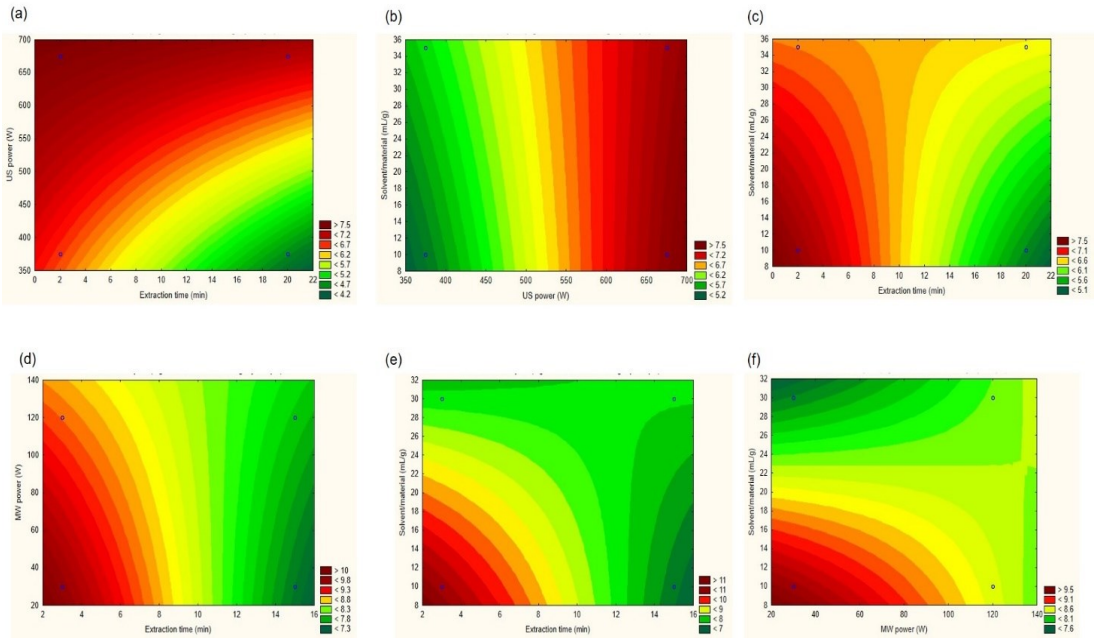



**Figure 6.10. Contour plots of $2^3$ design for UAE (a-c) and MAE (d-f) of shrimp body.**

ANOVA was employed to estimate the significance and suitability of the proposed model. The results of ANOVA are presented in Supplementary data, Table S10b.

After the omission of BBD models insignificant terms, the experimental data were fitted to the polynomial regression and the predicted models addressed to the data were expressed, in terms of coded values, by the following equations for UAE (Eq. 12) and for MAE (Eq. 13):

$$\text{UAE yield (mg of carotenoids 100 g}^{-1}\text{ dry sample)}=6.5841-0.1117x_1-0.5725x_1^2+0.4112x_2-0.1312x_2^2-0.9475x_3+0.4737x32-1.3675x_1x_2^2-0.0400x_1x_3-0.1612x_1^2x_3+0.3375x_2x_3 \quad \text{(Equation 12)}$$

$$\text{MAE yield (mg of carotenoids 100 g}^{-1}\text{ dry sample)}=9.3404-0.1837x_1^2+0.1725x_2-0.7286x_2^2+0.2755x_3+0.2631x_3^2+0.6310x_1x_2-0.4818x_1x_2^2-1.0871x_1^2x_2-0.3233x_1x_3-0.5723x_1^2x_3+1.1070x_2x_3 \quad \text{(Equation 13)}$$

According to ANOVA results for UAE, (a) the quadratic term of extraction time ($x_1^2$), (b) the linear term of US power ($x_2$), (c) the linear ($x_3$) and quadratic terms of solvent/material ratio ($x_3^2$) and (d) the interaction between the linear term of extraction time and the quadratic term of US power ($x_1x_2^2$) had a significant effect on the extraction yield. Respectively, (a) the quadratic term of extraction time ($x_1^2$), (b) the interaction between the quadratic term of time and the linear

term of MW power ($x_1^2x_2$) and (c) the interaction between the linear terms of time and solvent/material ratio ($x_1x_3$) were found significant in MAE. The importance of each factor can be illustrated graphically with the Pareto chart of standardized effect for UAE and MAE in Figure 6.11.

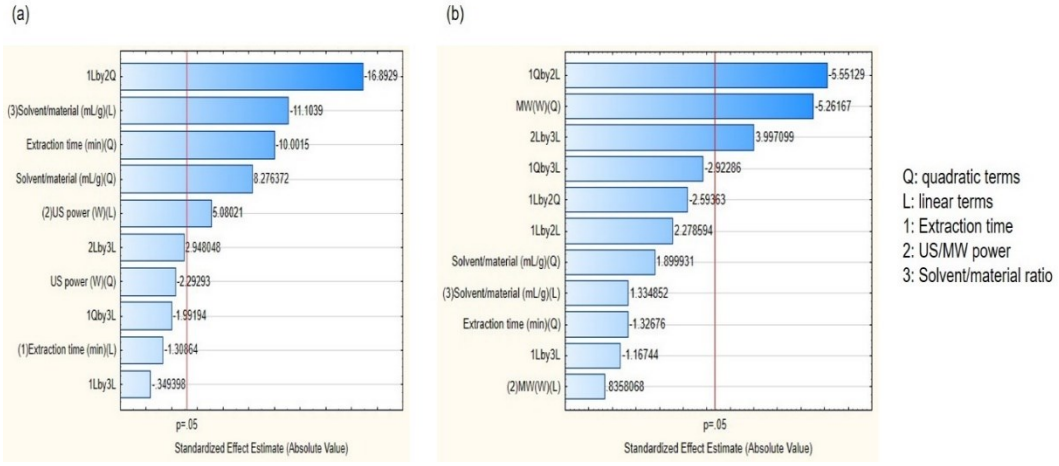



**Figure 6.11. Pareto charts for (a) UAE and (b) MAE of shrimp body.**

The proposed models showed accuracy and good fit, as the values of determination coefficient $R^2$ for both techniques ($R^2$=0.996, for UAE and $R^2$=0.968, for MAE) indicated an important agreement between the observed and the predicted, by the quadratic models, values. The adjusted determination coefficient $R^2_{adj}$ verified the adequacy of the model, as its value was higher than 0.80 in both cases. In particular, more than 98.7% and more than 88.0% of the response variation was explained, implying an accurate model for UAE and MAE, respectively. Lack of fit p-values are higher than the significance level of 0.05 (UAE p-value=0.99 and MAE p-value=0.58), therefore our models fit excellent to the experimental data (Supplementary data, Table S10b). Furthermore, repeatability and reliability of BBD models were confirmed by low standard deviation values of the four replicates at central points (0,0,0) (Standard deviation UAE=0.23, Standard deviation MAE=0.65).

Figures 6.12 depicts the response surface plots of UAE (Fig. 6.12a–c) and MAE (Fig. 6.12d–f).

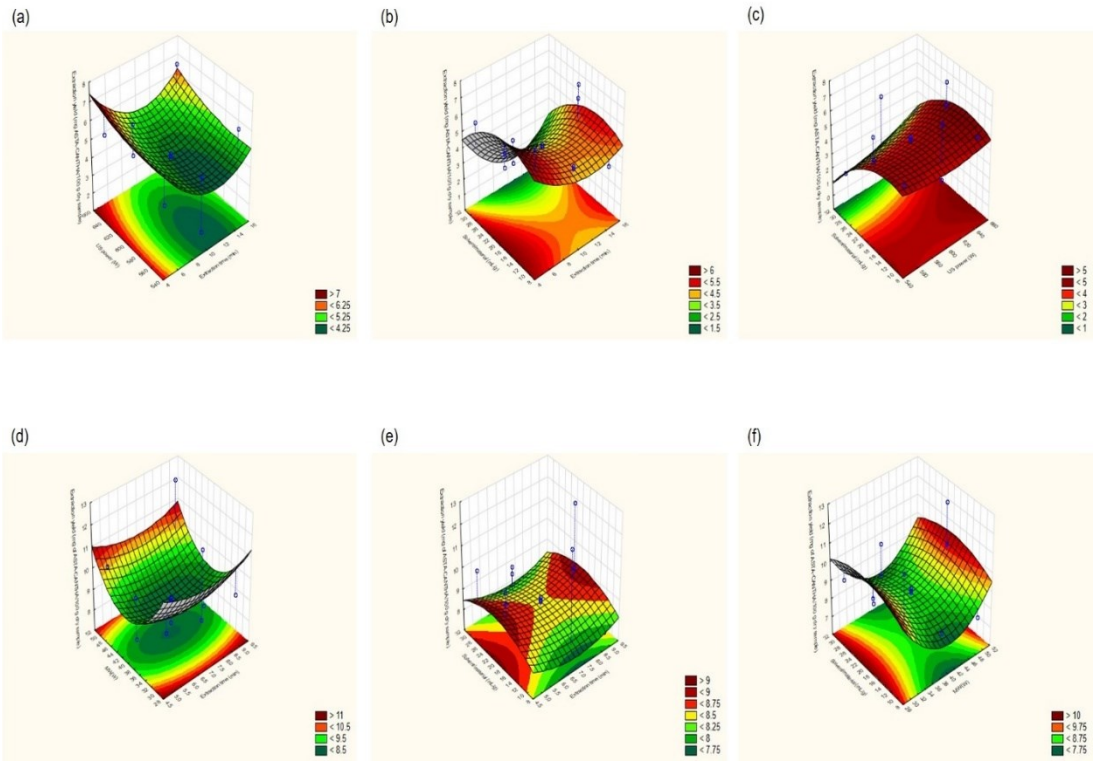



**Figure 6.12. RSM plots for UAE (a-c) and MAE (d-f) of shrimp body.**

## i) Effect of extraction time

Figures 6.12a and 6.12b demonstrate the interaction of extraction time with US power and solvent/material ratio, respectively. As it is shown in these figures, the higher extraction yield should be expected at time values lower than 10 min, when US power was set at high values and solvent/material ratio at medium ones. Shorter extraction time were preferred as it protect carotenoids from a possible oxidative degradation caused by the prolonged US irradiation [120, 145].

Figures 6.12d and 6.12e show the effect of extraction time in relation to MW power and solvent/material ratio on carotenoid yield. As it can be deduced from the response surface plots, the investigated system seems to perform better either at high or low extreme values of extraction time. Nevertheless, in short time periods, the solvent mixture was not sufficiently heated, a fact which may cause an incomplete recovery of carotenoids from the matrix. Longer exposure times (over 10 min) may affect the stability of carotenoids and decrease the extraction yield [146].

**ii) Effect of US/MW power**

Since the instrumental characteristics and operational power of the UAE and MAE apparatus differ, the range of the experimental values of US and MW power is different.

Figures 6.12a and 6.12c shows the effect of US power and its interaction with extraction time and solvent/material ratio. Both figures illustrated that an increase in the US power would result at higher extraction yield, due to the easier disruption of the tissues, which accelerates the diffusion of carotenoids and enhances mass transfer and extraction of target compounds [144]. Nevertheless, US power level higher than 750 W is not achievable, as this value is the upper extreme operational power of the UAE instrumentation used in this study. Furthermore, at extreme high values of US power, the extraction yield can be decreased, as the excessive increase in the size of cavitation bubbles cause the reduction of cavitation effect and possible scattering of US waves [145].

The effect of MW power on carotenoids extraction is demonstrated in Figures 6.12d and 6.12f. As it is obvious, extraction yield was maximized in low or either high values of the experimental range of MW power. Thus, due to energy-saving reasons and since the extraction yield of carotenoids was high in low MW power there was no point in directing the experimental design model to higher power values [146].

**iii) Effect of solvent/material ratio**

Figures 6.12b and 6.12c presents the influence of solvent/material ratio on UAE of xanthophylls. From these figures, it can be concluded that the higher carotenoid yield could be achieved at medium and low values of solvent/material ratio. Since higher ratio did not seemed to improve the extraction yield crucially, lower solvent/material ratios provided efficient extraction yields and low solvent consumption [145].

According to Figures 6.12e and 6.12f, in MAE, a medium level of solvent/material ratio led to a satisfactory extraction yield, because solvent volume was enough to keep the matrix immersed throughout all extraction

procedure and at the same time, at a medium level of solvent/material ratio the exposure and distribution of MW is more uniform than in a higher level [146].

**iv) Optimal extraction conditions**

The interpretation of RSM plots (Fig. 6.12) resulted in three experimental runs around the optimum area of the experimental range in order to determine the exact optimal combination of extraction conditions (Supplementary data, Table S11).The predictive capability of the established models is presented in the plots of observed versus predicted values (Supplementary data, Figure S5).

To summarize the results of the BBD optimization for shrimp body, the **optimal values** of UAE and MAE are presented in Table 6.6.

**Table 6.6. Optimal values of UAE and MAE parameters for carotenoids recovery from shrimp body and head.**

| Extraction parameters | Optimal values | |
|---|---|---|
| | **UAE** | **MAE** |
| **Extraction solvent (v/v)** | Acetone | n-Hexane:Acetone:Ethanol 2:1:1 |
| **Extraction time (min)** | 5 | 7 |
| **US/MW power (W)** | 600 | 30 |
| **Solvent/material ratio (mL $g^{-1}$)** | 10 | 20 |
| **US pulse sequence (s)/MW ramping time (min)** | 15 ON 5 OFF | 0 |
| **Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) of shrimp body (±stdev), n=3[a]** | 6.73(±0.56) | 13.3(±1.1) |
| **Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) of shrimp head (±stdev), n=3[a]** | 23.4(±2.3) | 23.92(±0.63) |

[a*]n: number of sample replicates measured under repeatability conditions

## 6.3 Replacing organic conventional solvents with green natural deep eutectic solvents (NADES)

The sustainable large-scale utilization of bioactive extracts from agro- and food byproducts requires the substitution of the organic conventional solvents with novel extractant medium, such as natural deep eutectic solvents. Thus, after

optimizing UAE and MAE conditions for the recovery of carotenoids from apricots pulp and shrimp head, we proceeded with the replacement of the optimal solvents with a 2:1 choline chloride:tartaric acid (CC/TA) natural deep eutectic solvent (NADES). Besides extraction solvent, the rest of the extraction parameters were set at the optimal values delivered by BBD models.

### 6.3.1 NADES NMR characterization

2D NMR experiments are often used in order to investigate the spatial coordination of NADESs [99]. In this work, the intermolecular assembly between CC/TA was investigated through $^{1}H$-$^{1}H$ Nuclear Overhauser Effect (NOESY) spectroscopy in DMSO-$d_6$ showing protons in spatial vicinity (Supplementary data, Figure S6) [177].

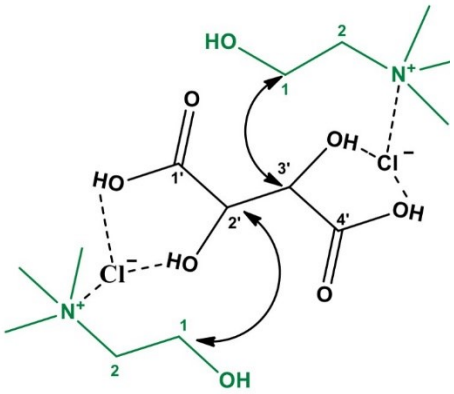



**Figure 6.13. NOESY intermolecular proton interactions of NADES [177].**

The spectra of CC/TA show broadened peaks, which are characteristic for such systems, due to the occurrence of dipolar interactions (inter- and intramolecular) (Bubalo et al, 2015). In our study, the NOESY spectra showed a strong correlation between the C1 protons of choline chloride with the C2´ and C3´ protons of tartaric acid. The hydroxyl groups of tartaric acid seem to interact with each other without affecting other protons, which could be an indication of strong hydroxyl attraction from the chlorine anion. According to the work of Abbott et. al. (2004) [208], the chlorine anion is capable of forming two hydrogen bonds with hydroxyl groups, which also comes in agreement with the molecular coordination proposed in Figure 6.13 with two choline chloride molecules interacting with one molecule of tartaric acid [208]. Moreover,

NOESY spectrum shows intramolecular correlation between the C2 and the hydroxyl group protons of choline chloride.

### 6.3.2 NADES thermal properties

DESs tend to have slightly modified thermal properties in comparison to their individual components, due to the formation of a dense hydrogen bond network. This theory was proved by performing TGA and DSC experiments on the physical mixture of the components (Supplementary data, Figure S7a), as well as CC/TA NADES (Supplementary data, Figure S7b) [177].

The physical mixture of choline chloride and tartaric acid shows some easily interpreted TGA and DSC spectra. The endothermal DSC peak at 80 °C can be attributed to the existence of hydrogen bonds between the compounds, signaling the NADES' formation. This observation comes in agreement with the NADES synthesis procedure, as the compounds are heated at 80ºC for specific time. Therefore, it is obvious that for the following experiment, we study the properties of an intermediate system, between the NADES and the physical mixture. However, it is expected that in the short period of time that is required for this experiment, just a small percentage of the compounds has been turned into the NADES, as the full homogenization of the mixture requires about 4 h. The endothermal peaks, at 205 and 265 ºC could be attributed to the degradation of tartaric acid and choline chloride respectively, taking into consideration the weight loss percentage in each case [177].

CC/TA shows slightly modified spectra in comparison to the physical mixture. First of all, there is no endothermal peak at 80ºC, meaning that the hydrogen bond system has been fully formed. The degradation temperatures of tartaric acid and choline chloride have been shifted to 220 and 280ºC respectively. This observation can be easily explained, by assuming that the system requires more energy for the degradation, as hydrogen bonds need to be disrupted first. This differentiation in the energy requirement is translated in degradation temperature rising by 15 ºC for both components [177].

### 6.3.3 UAE and MAE of apricot byproducts and shrimp head using NADES

After optimizing high energy extraction conditions for the recovery of carotenoids from apricot pulp and shrimp head using BBD and conventional solvent system, we implemented these results to CC/TA-based extraction in order to compare the extraction yields of organic and NADES solvents. Optimal conditions for each case are mentioned in Sections 6.2.1 and 6.2.3. A 1:1 (v/v) chloroform-methanol mixture was applied for conventional UAE in apricots pulp, while extraction solvent of shrimp head UAE was acetone. MAE organic solvent system was ethanol and a 2:1:1 (v/v/v) mixture of n-hexane:acetone:ethanol for apricots and shrimps, respectively [146].

The comparative results of conventional and NADES solvents are presented in Table 6.7.

**Table 6.7. Carotenoids extraction yields of conventional and NADES solvents.**

| Extraction solvent (v/v) | Apricot pulp extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev, n=3)* | Shrimp head extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev, n=3)* |
|---|---|---|
| **UAE-conventional solvents** | 11.12(±0.34) | 23.4(±2.3) |
| **UAE-NADES** | 41.3(±1.9) | 7.85(±0.47) |
| **MAE-conventional solvents** | 26.5(±2.2) | 23.92(±0.0.63) |
| **MAE-NADES** | 76.11(±0.84) | 26.7(±2.5) |

According to Table 6.7, NADES extraction seems to provide significantly higher carotenoids yield in most cases, compared to conventional solvents. This could be related to the nature of the matrix and its affinity to NADES.

CC/TA NADES is a polar nature solvent while shrimp head is a lipid-rich natural substrate [209]. Therefore, at UAE's short extraction time (5 minutes), acetone proved to be a more efficient extraction solvent for shrimp waste. According to literature, UAE-NADES of astaxanthin requires longer extraction time and higher solvent/material ratio [210]. On contrary, in MAE's case, despite the short extraction duration (7 minutes), NADES achieved carotenoids yield similar to organic solvents' due to the increased extraction temperature (35 °C in UAE and 52 °C in MAE). In general, temperatures around 45-50 °C enhance

extraction yields due to the improved diffusivity and permeability of NADES caused by its reduced viscosity and surface tension [211].

On the other hand, carotenoids yield in apricots byproducts, was essentially higher in both UAE and MAE, when NADES was used as solvent. Apricots do not contain significant amounts of lipids, thus NADES could be a more adequate extraction medium for apricots than shrimp wastes. In addition, optimal extraction time was longer (10 minutes for UAE and 20 minutes for MAE) [211]. The combinatorial effect of NADES extraction strength, US/MW power, longer extraction periods and extraction temperature, in the case of MAE, was reflected on the extraction yields, which were 4-times higher in UAE and 3.5-times higher in MAE compared to conventional systems. Although carotenoids' yields depend on various factors (i.e. cultivar, geographical origin, climate), NADES succeeded to extract similar or even higher carotenoids content compared to conventional organic solvents, especially when MAE was applied [29].

# CHAPTER 7
# DEVELOPMENT OF A LIQUID CHROMATOGRAPHY-TANDEM MASS (LC-MS) SPECTROMETRY METHOD FOR THE ANALYSIS OF CAROTENOIDS

Hyphenation of DOE results with high throughput analytical techniques as liquid chromatography-mass spectrometry (LC-MS) forges a powerful tool for a detailed analysis of complex matrices. In the current project, DOE models based on spectrophotometric measurements provide a quantitative estimation of carotenoids content in optimal extraction values. Nonetheless, liquid chromatography-tandem mass spectrometry using atmospheric pressure chemical ionization (APCI(+) LC-MS/MS) give information about the exact profile of carotenoids of optimized MAE and UAE extracts [212]. In addition, a photodiode array (PDA) detector was used for the observation and identification of carotenoids isomers.

## 7.1 Constructing matrix-matched calibration curves

Matrix-matched calibration curves (carotenoids area ratio vs μg $mL^{-1}$) [213, 214] were used for carotenoids quantification. From each substrate, a pooled mix from UAE and MAE extracts, respectively, was used for calibration curves construction.

As stated in Section 5.8, samples containing 20 mg of apricots extract, 100 mg of egg yolk extract, 40 mg of shrimp head and 20 mg of shrimp body were spiked with standard solutions of carotenoids in order to prepare samples with final concentrations of added standards of 0.5–20 μg $mL^{-1}$. Final values are expressed in mg 100 $g^{-1}$ of dry egg yolk. Meanwhile, calibration curves of carotenoids standards at the above concentration range, dissolved only in injection solvent (neat samples), were also constructed. Calibration curves used for quantitation purposes were those resulted from matrix-matched process. Any possible carry-over effects were eliminated by injecting and running blank solvent samples.

Since isotopically labeled standards skyrocket analysis cost and the possibility of obtaining a carotenoid-free blank matrix is extremely difficult, we applied the

matrix-matched calibration curve strategy using *trans*-β-apo-8′-carotenal, an oxocarotenoid, as internal standard (IS) throughout all validation steps to counteract potential experimental errors and variations. Internal standard (IS) concentration in all samples was 1 μg $mL^{-1}$.

## 7.2 Fragmentation pattern of carotenoid standards

Identification and quantification of target carotenoids (b-carotene, lutein, zeaxanthin, astaxanthin and canthaxanthin) was conducted by selected-reaction monitoring (SRM) mode. The mass transitions that served as diagnostic m/z ions for each compound are presented in Table 7.1.

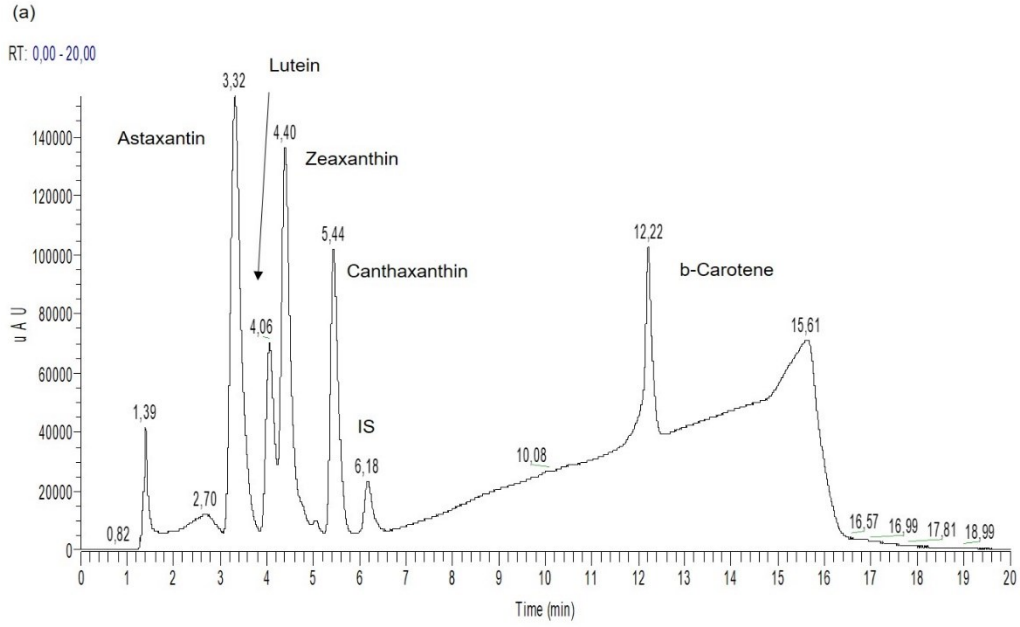
**Table 7.1. APCI(+) LC-PDA-MS/MS characteristics of carotenoids.**

| Carotenoid standards | RT (min) | $\lambda_{max}$ (nm) | Parent ion (m/z) $[M-H]^+$ | Product ion used for quantification (m/z) $[M+H-yA]^+$ |
|---|---|---|---|---|
| **b-Carotene** | 12.22 | 453 | 537.4 | 444.3 $[M-C_7H_8]^+$ |
| **Lutein** | 4.03 | 446 | 569.4 | 533.3 $[M+H-2H_2O]^+$ |
| **Zeaxanthin** | 4.41 | 452 | 569.4 | 551.3 $[M+H-H_2O]^+$ |
| **Astaxanthin** | 3.29 | 478 | 597.4 | 579.3 $[M+H-H_2O]^+$ |
| **Canthaxanthin** | 5.63 | 476 | 565.4 | 547.3 $[M+H-H_2O]^+$ |
| **IS** | 6.16 | 464 | 417.4 | 399.3 $[M+H-H_2O]^+$ |

Since b-carotene is a totally non-polar compound, the formation of a protonated precursor ion is less favorable compared to xanthophylls. However protonated molecules of carotenes can be produced in APCI due to the promotion of π-electrons to higher excited states, which present high proton affinity after their interaction with APCI plasma. Moreover, the presence of MTBE in the elution system enhance the formation of a $M^+$ ions, while the use of methanol favors the generation of $MH^+$ ions. As both solvents were used in the mobile phase, the fragment ion produced is $[M-C_7H_8]^+$ (m/z=444.4), which match to toluene loss, typical for polyene chains [215].

Fragmentation pattern of isomer structures of lutein and zeaxanthin is the same, therefore the different intensities ratios of their product ions is the key for their identification. In the case of lutein, the intensity of product ion with m/z=551.4 is higher than that of parent ion of m/z=569.4, which, contrarily, is the most abundant fragment in zeaxanthin spectra. The differentiation on fragments intensity ratio is ascribed to i) the distinct number of molecules' chiral centers (three for lutein and two for zeaxanthin) ii) the position of –OH in structure rings (ε-ring in lutein and β-ring in zeaxanthin) iii) the stabilization of the ion formed from the elimination of water (m/z=551.4), which is induced the presence of –OH in the allylic position of lutein's ε-ring [216]. In addition, lutein is distinguished from zeaxanthin by fragment ion with m/z=495.3, which is observed only during the fragmentation of lutein and is a product of the loss of a water molecule from the protonated parent ion and the retro-Diels-Alder cleavage of a-ionone ring [217].

The loss of a water from hydroxycarotenoids and ketocarotenoids, like astaxanthin and canthaxanthin, produce the diagnostic ion with m/z=579.3 and m/z=547.3, respectively [216]. Likewise, the diagnostic ion of *trans*-β-apo-8′-carotenal is also the result of the loss of a water molecule (m/z=399.3) [216]. Retention times and mass transitions of carotenoids standards are presented in Figure 7.1.



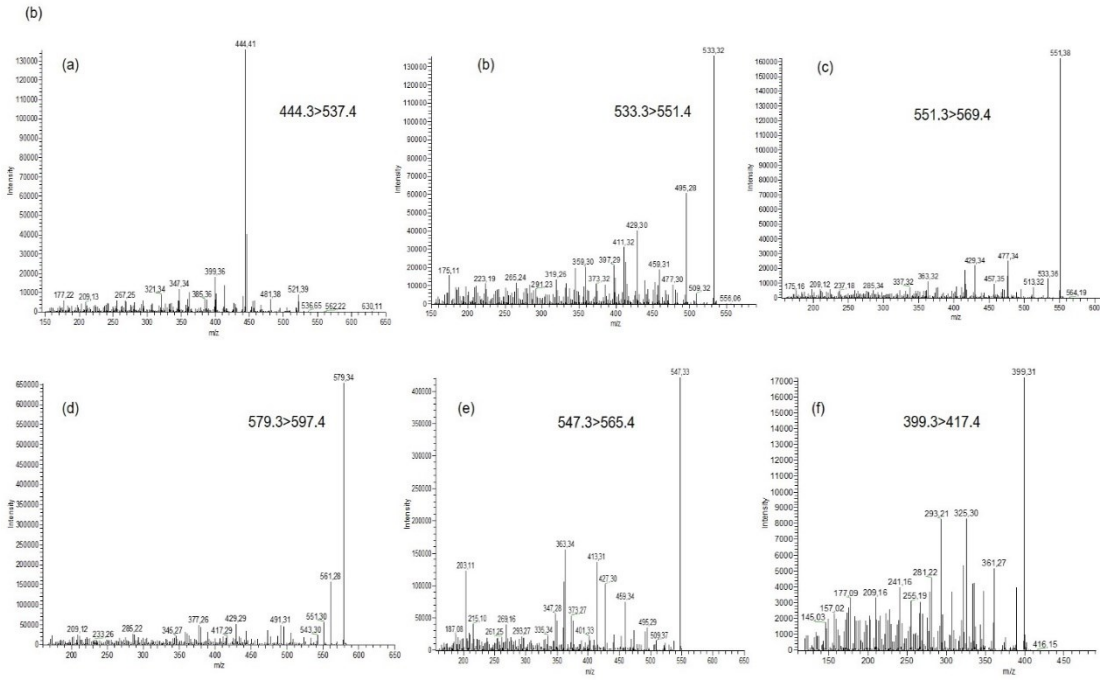



**Figure 7.1. (a) Retention times (b) mass transitions of carotenoids standards.**

## 7.3 Method validation

Matrix effect, linearity, precision, accuracy, recovery, limit of detection (LOD) and quantification (LOQ) were reviewed in our validated LC-MS/MS method. Detailed results of APCI(+) LC-MS/MS quantification are shown in Supplementary data, Tables S12 and S13.

### 7.3.1 Matrix effect (ME)

Determining the effect of matrix on LC-MS signal is of great importance during validation since matrix components could either suppress or enhance the signal of the target compounds compromising the analytical figures of merit of the developed method (precision, accuracy, LOD, LOQ, etc.) [218]. When the studied analytes are present in the matrix, ME could be estimated by the ratio of the slope of matrix-matched curve and the slope of neat samples calibration curve. ME calculated with this approach does not depend on analyte concentration, pre-existing in the extract or added as spiked standard. ME over 100% indicates enhancement of MS signal, while ME below 100% reveals MS signal suppression. Matrix effect of carotenoid for every substrate and extraction method are demonstrated in Supplementary data, Table S12a-c.

According to Table S12a a significant signal enhancement (ME(%)≥100) was observed both UAE and MAE carotenoids analysis [219]. Even though APCI is less susceptible to ME than ESI, many researchers observed signal enhancement and not suppression especially when neutral and generally non polar compounds were analyzed [220]. For example, egg yolk cholesterol and phospholipid content is considered responsible for increasing MS response [218]. The presence of significant MEs is probably ascribed to the lipid nature (egg yolk) or to the presence of sugars (apricots) and complexity of examined substrates, where co-extractives other than carotenoids could be ionized [221]. This last assumption is supported by the fact that in absence of sugars, for example in shrimp head and body, ME could reveal a signal suppression.

### 7.3.2 Calibration curves estimators

Calibration curves concentration ranged between 0.5-20 μg $mL^{-1}$. Regression coefficient ($R^2$) of carotenoids (Supplementary data, Table S12a-c) verified calibration curves linearity.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated by creating two additional calibration curves (one for UAE and one for MAE) at the area of lowest spiking levels of each carotenoid of all three substrates (Supplementary data, Table S12a-c) [222]. LOD and LOQ calculation were determined as $(3.3s_b/a)\times$IS concentration (μg $mL^{-1}$) and $(10s_b/a)\times$IS concentration (μg $mL^{-1}$), respectively.

In LOD and LOQ formulas, *a* stands for calibration curve slope, $s_b$ for intercept standard deviation and IS concentration is equal to 1 μg $mL^{-1}$.

In general, LODs/LOQs of b-carotene were higher than those of xanthophylls (Table S12a) due to the more intense background noise and matrix interferences at b-carotene elution. As it can be deduced from Table S12b, lutein could be quantified at lower concentration by UAE, while the opposite apply for zeaxanthin. Finally, astaxanthin and zeaxanthin were quantified at lower concentrations compared to canthaxanthin in shrimp body (Supplementary data, Table S12c). In shrimp head, LODs and LOQs of carotenoids are higher than in shrimp body due the high contents and possible

interferences of natural polymeric structures like chitin (Supplementary data, Table S12d) [223].

### 7.3.3 Precision, stability, accuracy, and recovery

Method precision was estimated in terms of intra- and inter-day repeatability. Three quality control (QC) samples at three different concentration levels (low, medium, high) were measured to calculate coefficient of variation (CV or RSDr(%)). Intra-day repeatability was carried out by running three replicates of the three QC samples on the same day. Inter-day repeatability was determined from the data obtained by running three replicates of QC samples at three different days. In line with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines [224], the developed LC-MS/MS method was precise as RSDr(%) of all QC samples were lower than 15% (Supplementary data, Tables S13a-c).

The study of carotenoids standards stability was performed by preparing the three QC samples of all carotenoids from the stock solutions kept in the refrigerator for one month and by comparing the results with those obtained from the LC-MS/MS analysis of QC samples on the first day of stock solution preparation. Based on *p*-values which revealed a statistically significant difference, the stock solutions of all carotenoids were degraded in one month period even though they were kept in dark and cold place.

Method trueness was expressed as percent relative error (RE%). The limits within a method is considered accurate are 80-110% for concentration levels under 100 µg $mL^{-1}$ and above 100 ng $mL^{-1}$ [225]. RE% was within the limits for all carotenoids standards in both extraction methods and substrates (Supplementary data, Tables S13a-c).

Process recovery is an indicator of the combined effect of matrix effect and extraction efficiency. Calculation of process recovery was determined by the equation (Eq.14) below:

Process recovery(%)=[(Peak area of pre-spiked sample-Peak area of analytes in unspiked sample)/Peak area of neat sample]*100 (Equation 14),

where pre-spiked sample is the sample with a spiked concentration of standards **before** the extraction step and neat sample is the sample of the

same concentration without the matrix [220]. Although recovery values in the range of 70-120% are generally considered satisfactory, process efficiency is acknowledged as valid when recovery values are (i) precise (ii) reproducible and (iii) over the threshold of 20% (ICH guidelines) [224]. Recovery values for all samples are presented in Tables S13a-c.

As presented in Table S13a, UAE recovery values were almost equal for b-carotene and lower for zeaxanthin and lutein, compared to MAE. According to Song et al (2015) [226], in a model system, the conversion of *trans*-lutein to 13-*cis* lutein, 13′-*cis* lutein, 9-*cis* lutein and 9′-*cis* lutein follows a second-order kinetics at ~30 ºC. Thus the lower UAE recovery of xanthophylls compared to b-carotene could be assigned to their more increased degradation rate under US treatment [226].

Nonetheless, MW treatment is responsible for xanthophylls isomerization but its extend depends on the interrelation of applied MW power, extraction time and the nature of investigated substrate. A possible explanation for MAE recovery values (Supplementary data, Tables S13a-c) might be attributed to a degradation of free forms of carotenoids from the effect of MWs which provoke partial loss of all-*trans* lutein, from its conversion to 13-*cis*-lutein and zeaxanthin isomers [227]. In the case of shrimps, where extraction time is short in both UAE and MAE, but the applied energies differs significantly (30 W in MAE and 600 W in UAE), the recoveries of zeaxanthin in MAE are higher (Supplementary data, Table S13c-d).

Ultrasound (US)- and microwave (MW)- speed up the degradation of ketocarotenoids (i.e. astaxanthin, canthaxanthin) (Supplementary data, Tables S13c-d) even at short extraction times (≤6 min) producing 9- and 13-*cis*-isomers when MW is applied and colorless compounds when US was implemented (Fig. 7.2) [228, 229].

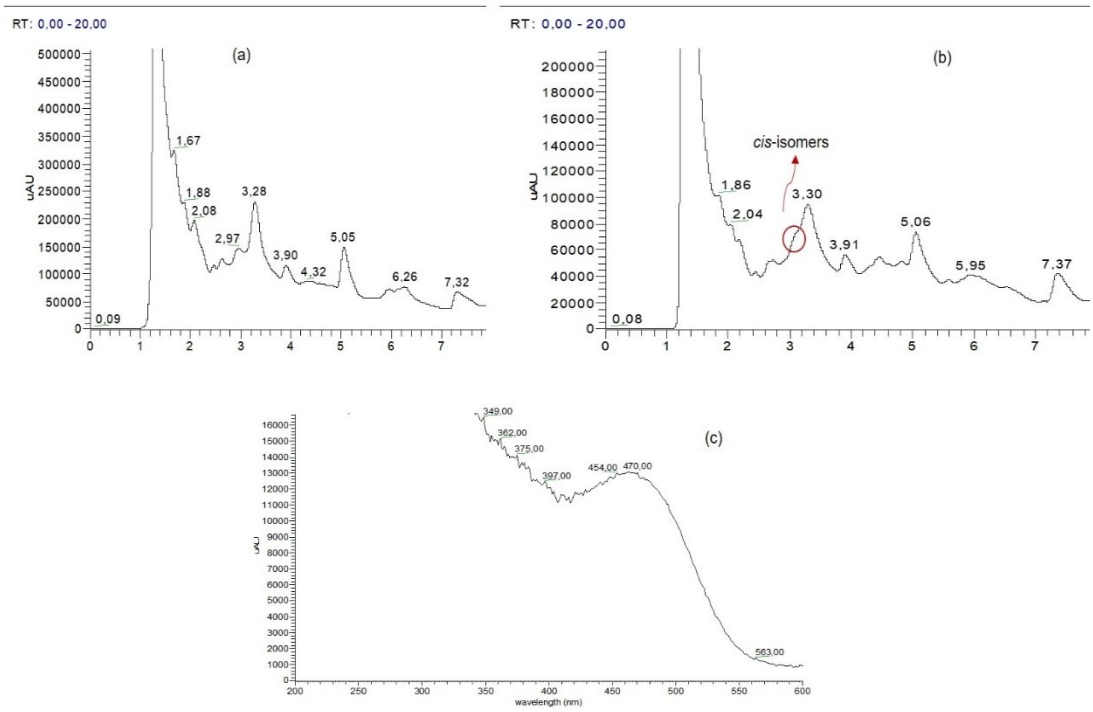



**Figure 7.2. PDA chromatograph for (a) UAE, (b) MAE of shrimp body and (c) spectrum of MAE cis-isomers.**

Although carotenoids are more susceptible to *cis*-transformation under MW treatment [41], in the current work their isomerization is also present when UAE was applied possibly due to significantly higher applied US power compared to MW power. The extend of this phenomenon is multifactorial and cannot be generalized for all cases, as it depends on the applied extraction conditions, especially temperature, the US/MW power, the stability of quantified carotenoids and the nature of examined substrates. Overall, outlier recovery values could be ascribed to matrix interferences and lack of suitable isotopically labeled standards.

Compared to other methodologies in the literature [230, 231] our validated LC-MS/MS methodology managed to identify and quantify reliably five important carotenoids by using a short gradient program. Therefore, the present analysis achieved to separate adequately and faster than other currently available protocols some of the most common and valuable carotenoids of natural substrates.

## 7.4 Optimized UAE and MAE: which one provides higher extraction yield?

The quantitation of carotenoids would give an answer to the question: Which extraction technique is more ideal to recover carotenoids from plant or seafood byproducts and lipid food matrices?

### 7.4.1 Apricot byproducts

The results delivered from LC-MS/MS analysis for the carotenoid content of apricot pulp are presented Table 7.2.

Based on the results of Table 7.2, three carotenoids were quantified in apricot byproducts, **1) b-carotene, 2) zeaxanthin and 3) lutein** (Supplementary data, Figure S8).

MAE extracted almost 2.5-times more b-carotene compared to UAE. On the other hand, Folch method did not stick out as an apt choice for b-carotene recovery from fruit tissues since b-carotene content was 19-fold and 7.5-fold lower than in MAE and UAE, respectively. The outcome of LC-MS/MS method is probably related to (a) the high polarity of ethanol which allow the absorbance of MW energy and the acceleration of MAE process, and (b) the combinatorial effect of MW power and increased (compared to UAE) extraction temperature, which cause looseness and disruption of the tight cell structure and enhance diffusion of b-carotene in ethanol [162]. In parallel, recent studies [226] reported the US degradation of b-carotene to *cis*-isomers, oxygenated derivatives and β-apo-carotenals (i.e. 15-Z-b-carotene, di-Z-b-carotene, 9-cis-b-carotene) due to isomerization, oxidation and cleavage reactions. The deterioration of *trans*-b-carotene to these derivatives could explain the lower UAE yield.

**Table 7.2. Carotenoid content of apricot pulp determined by LC-MS/MS.**

| **Carotenoids content (mg 100 g$^{-1}$ dry sample), v=3[a]** | **Optimized UAE extract** | **Optimized MAE extract** | **Folch extract** |
|---|---|---|---|
| b-Carotene | 7.72(±0.98) | 19.7(±1.6) | 1.44(±0.87) |
| Zeaxanthin | 0.71(±0.33) | 0.66(±0.25) | 0.020(±0.041) |
| Lutein | 0.82(±0.19) | 0.82(±0.12) | 0.07(±0.24) |
| **Final carotenoids content expressed in mg kg$^{-1}$ of raw apricot pulp sample (N=3)[b]** | | | |
| **Average weight (g) of raw apricot pulp samples (±stdev), n=10[c]** | 17.1(±2.1) | | |
| **Average weight (g) of lyophilized apricot pulp samples (±stdev), n=10[c]** | 3.23(±0.39) | | |
| **Average (%) moisture of raw apricot pulp, n=10 [c]** | 81.25 | | |
| | **UAE extracts** | **MAE extracts** | **Folch extract** |
| **b-Carotene content (mg kg$^{-1}$ raw apricot pulp) (±stdev)** | 14.58(±0.98) | 37.2(±1.6) | 2.72(±0.87) |
| **Zeaxanthin content (mg kg$^{-1}$ raw apricot pulp) (±stdev)** | 1.34(±0.33) | 1.25(±0.25) | 0.038(±0.041) |
| **Lutein content (mg kg$^{-1}$ raw apricot pulp) (±stdev)** | 1.55(±0.19) | 1.55(±0.12) | 0.13(±0.24) |

*[a]:Number of LC-MS/MS replicates; [b]:Number of extraction replicates; [c]:Number of samples*

PDA chromatograph confirms the presence of *cis*-isomers in apricot byproducts (Fig. 7.3).

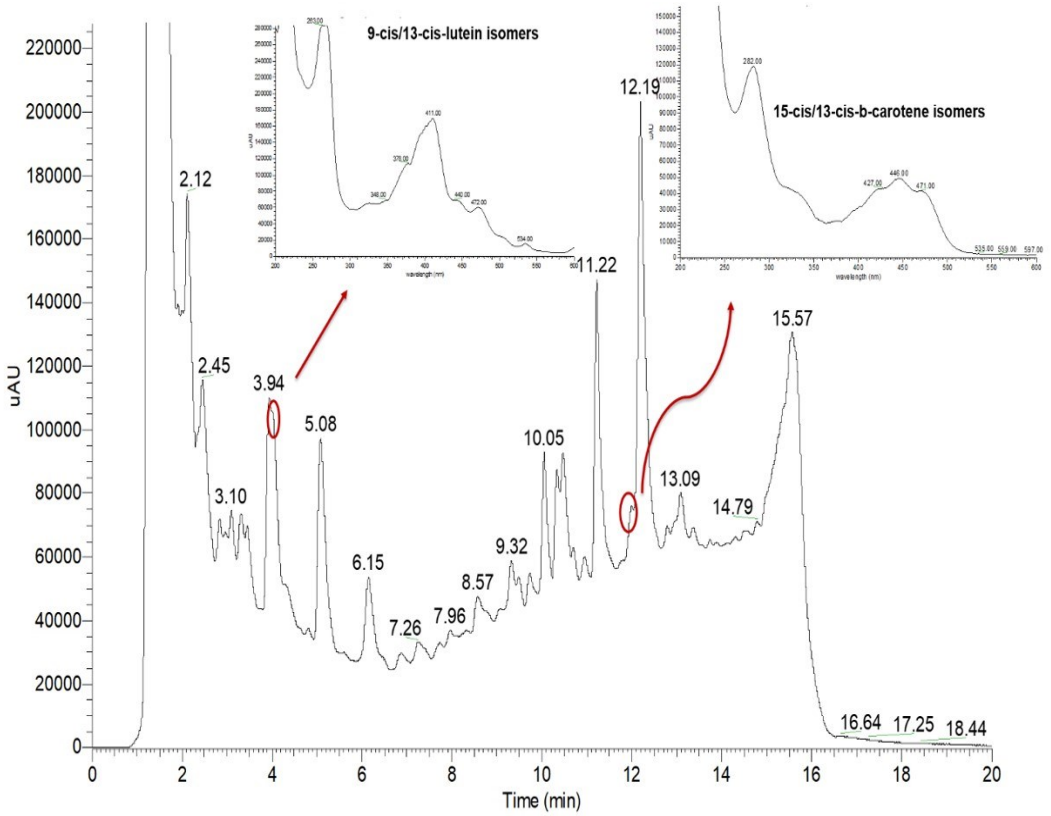



**Figure 7.3. PDA chromatograph for UAE of apricot pulp.**

The same trend was also observed for apricot xanthophylls content of classic extraction and high energy methods. Folch method recovered a minor amount of zeaxanthin and lutein, one order of magnitude lower than the xanthophylls content of high energy techniques. In contrast to the significant difference in b-carotene concentration, UAE and MAE extracted equal amounts of zeaxanthin and lutein (Table 7.2). Likely, similar xanthophylls yields may be assigned to the more extensive rate of US isomerization occurring in UAE [226].

Content of carotenoid-rich fruits and fruit byproducts, like apricots, presents important variations depending on the variety, cultivar and hybrids, geographical origin, climatic differences, genotype, ripening and development stages. Hence, evaluating the yields provided by different extraction techniques is pretty much a multifactorial task. Nevertheless, current MAE process managed to extract 4-times more b-carotene but similar xanthophylls equivalents compared to accelerated solvent extraction (ASE), while UAE and ASE resulted almost in the same final carotenoid content [157]. Classic extraction, applied in different varieties of apricots cultivated in New Zealand

and USA, achieved b-carotene yield close to MAE. Higher yields of lutein obtained by [232] method may support the hypothesis of xanthophylls isomerization under certain conditions of high energy extractions [29, 232]. In short, our research results enrich the general knowledge concerning the future large-scale implementation of UAE and MAE for the recovery of bioactive compounds from plant byproducts [233].

So, in terms of b-carotene yield, **MAE** was the ideal option for recovering carotenoids from a plant byproduct substrate, especially of seasonal fruit, like **apricot pulp**, with significant sugar content.

### 7.4.2 Egg yolk

The results delivered from LC-MS/MS analysis for the carotenoid content of egg yolk are presented Table 7.3. Two xanthophylls, **1) lutein and 2) zeaxanthin** were determined in egg yolk extracts (Supplementary data, Figure S9).

Higher US power enhanced extraction yield due to induced shear forces which improves granules penetration and mass transfer compared to other methods [8]. Therefore, UAE provided higher lutein and zeaxanthin yields (Table 7.3), an outcome that is consistent with findings from other studies [192]. Notwithstanding, UAE could achieve considerably higher yields (over 3-fold higher) at only 20 min extraction time compared to the 24 h needed for Folch extraction (Table 7.3). This could be ascribed to the fact that UAE proceed in two phases. In phase one, granule disruption and carotenoids release is more severe and rapid, while in phase two, extracted molecules are diffused through the extractant. Thus, a 20 min extraction duration is considered ideal to achieve efficient extraction yields without wasting time and energy [234].

**Table 7.3. Carotenoid content of egg yolk determined by LC-MS/MS.**

| **Carotenoids content (mg 100 g$^{-1}$ dry sample), v=3[a]** | **Optimized UAE extract** | **Optimized MAE extract** | **Folch extract** |
|---|---|---|---|
| Lutein | 1.7(±1.1) | 2.40(±0.77) | 0.36(±0.28) |
| Zeaxanthin | 4.5(±1.4) | 1.70(±0.69) | 1.532(±0.093) |
| **Final carotenoids content expressed in µg 20 g$^{-1}$ of raw egg yolk sample (N=3)[b]** | | | |
| **Average weight (g) of raw egg yolk samples (±stdev), n=10[c]** | 12.74 (±2.93) | | |
| **Average weight (g) of lyophilized egg yolk samples (±stdev), n=10[c]** | 8.91 (±1.24) | | |
| **Average (%) moisture of raw egg yolk, n=10 [c]** | 30.06 | | |
| | **UAE extracts** | **MAE extracts** | **Folch extract** |
| **Lutein content (µg 20 g$^{-1}$ raw egg yolk) (±stdev)** | 215.8(±1.1) | 269.7(±0.77) | 58.3(±0.28) |
| **Zeaxanthin content (µg 20 g$^{-1}$ raw egg yolk) (±stdev)** | 653.4(±1.4) | 280.8(±0.69) | 233.3(±0.093) |

*[a]:Number of LC-MS/MS replicates; [b]:Number of extraction replicates; [c]:Number of samples*

MAE outcome could be attributed to the synergistic effect of both MW power and extraction temperature. Lutein content of MAE was higher than the Folch method (over than 4-fold higher) (Table 7.3). Nevertheless, according to our results granules rupture of a lipid substrate, like egg yolk, required high extraction power to release more carotenoids. In addition, compared to UAE, MAE took place in higher temperatures, which can increase the amount of their 13-*cis*-isomers instead of their *trans*-analogs (Fig. 7.3) [41, 227]. This result could be supported by the fact that both in UAE and Folch the ratio of *trans*-lutein:*trans*-zeaxanthin stayed similar (around 1:3–1:4), while in MAE xanthophylls ratio was 1:1 (Table 7.3).

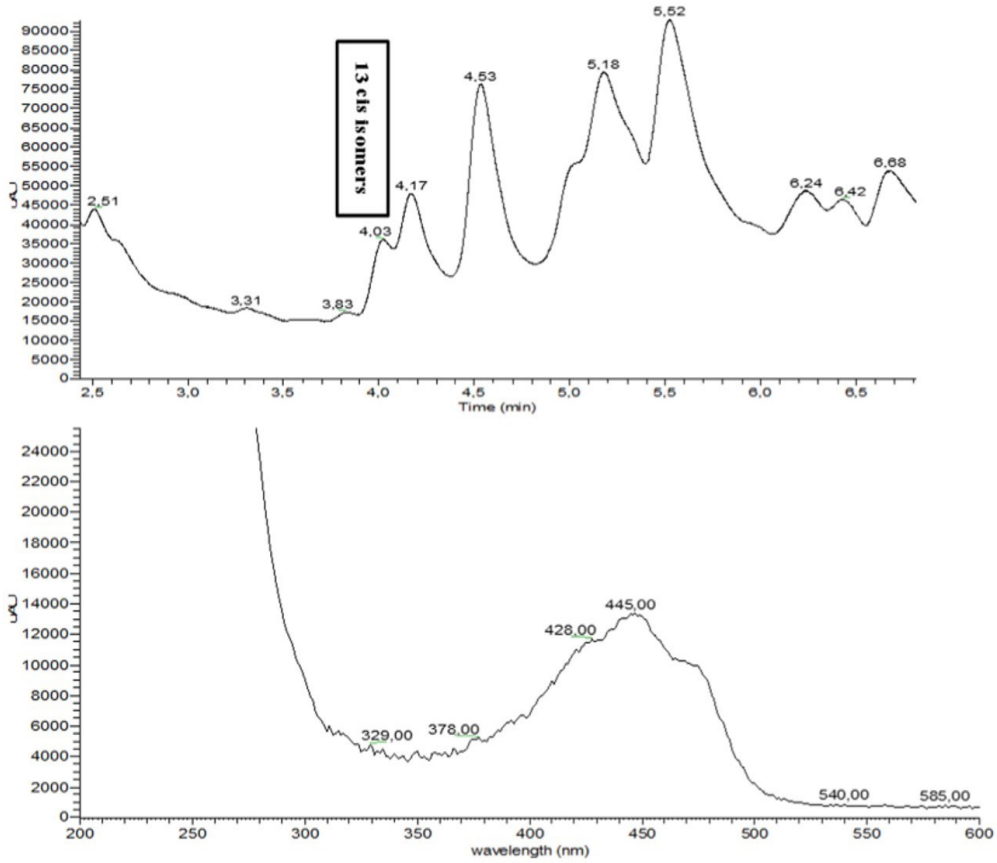



**Figure 7.4. 13-cis-isomers identification in PDA chromatograph of MAE extract.**

In addition, traces of another feed additive xanthophyll, canthaxanthin, were also detected (Supplementary data, Figure S10). As mentioned before, canthaxanthin is an approved synthetic additive for the adjustment of the egg yolk color, permitted at concentrations ≤66 mg/kg of solid or semisolid food and ≤4.41 mg/kg for broiler chicken [41].

As it has already been discussed, final content of egg yolk xanthophylls could differ crucially and therefore a reliable comparison of our developed high energy processes with the results of other groups is not always feasible. Although we managed to extract more xanthophylls than other reported studies [235], the most unbiased approach is to compare our data with the average content of xanthophylls in egg yolk. Egg yolk of a medium size egg weighs around 20 g, contains 200–400 μg of xanthophylls [40]. This amount of egg yolk xanthophylls was obtained by the 24-hours classic extraction method applied. According to Table 7.3 data, MAE method recovered successfully amounts of lutein and

zeaxanthin higher of that range. However, higher MAE temperatures could lead to partial degradation of xanthophylls.

On the other hand, UAE technique accomplished to extract quantities of lutein and zeaxanthin higher that the reported. Cavitational effects caused by higher US power and lower UAE temperatures succeeded to release carotenoids bound in different fatty acids structures by avoiding possible degradation reactions [202]. In details, bubbles produced by acoustic cavitation generated severe changes in mass medium pressure. The abrupt increase and decrease of pressure along with the minimization of egg yolk particle size by US disintegration increase the surface and the number of granules, which could be subjected to UAE [234].

Thus, **UAE** emerged as the most adequate technique to extract xanthophylls form a highly lipid matrix, like **egg yolk**. Nonetheless, attention should be paid to the acquisition of carotenoids extracts free from other matrix components. In the case of lipid substrates, like egg yolk, a tentative profile provided by NMR spectroscopy revealed that the main matrix interferences are lipids (Supplementary data, Figure S11). Although, the complete removal of other lipidic constituents could be a quite difficult and time-consuming task, SPE clean up protocols could be applied for the purification of carotenoids [236, 237].

### 7.4.3 Shrimp head and body

The results delivered from LC-MS/MS analysis for the carotenoid content of shrimp head and body are presented Table 7.4a-b. Based on these results, the concentration of four polar carotenoids were calculated in shrimp head and body: **1) astaxanthin, 2) canthaxanthin 3) zeaxanthin and 4) lutein** (Supplementary data, Figures S12 and S13).

**Table 7.4. Carotenoid content of (a) shrimp head and (b) shrimp body determined by LC-MS/MS**

**(a)**

| **Carotenoids content (mg 100 $g^{-1}$ dry sample), v=3[a]** | **Optimized UAE extract** | **Optimized MAE extract** | **Folch extract** |
|---|---|---|---|
| Astaxanthin | 18.74(±0.81) | 17.52(±0.94) | 8.50(±0.63) |
| Canthaxanthin | 1.59(±0.52) | 2.26(±0.44) | 0.86(±0.37) |
| Zeaxanthin | 0.71(±0.23) | 1.22(±0.51) | 0.61(±0.14) |
| Lutein | 0.82(±0.19) | 0.77(±0.21) | 0.34(±0.11) |
| **Final carotenoids content expressed in mg 100 $g^{-1}$ of raw shrimp head sample (N=3)[b]** | | | |
| **Average weight (g) of raw shrimp head samples (±stdev), n=10[c]** | 18.1(±2.6) | | |
| **Average weight (g) of lyophilized shrimp head samples (±stdev), n=10[c]** | 4.06(±0.93) | | |
| **Average (%) moisture of raw shrimp head, n=10 [c]** | 77.56 | | |
| | **UAE extracts** | **MAE extracts** | **Folch extract** |
| **Astaxanthin content (mg 100 $g^{-1}$ raw shrimp head) (±stdev)** | 4.20(±0.81) | 3.93(±0.94) | 1.91(±0.63) |
| **Canthaxanthin content (mg 100 $g^{-1}$ raw shrimp head) (±stdev)** | 0.36(±0.52) | 0.51(±0.44) | 0.19(±0.37) |
| **Zeaxanthin content (mg 100 $g^{-1}$ raw shrimp head) (±stdev)** | 0.16(±0.23) | 0.27(±0.51) | 0.14(±0.14) |
| **Lutein content (mg 100 $g^{-1}$ raw shrimp head) (±stdev)** | 0.18(±0.19) | 0.17(±0.21) | 0.08(±0.11) |

*[a]:Number of LC-MS/MS replicates; [b]:Number of extraction replicates; [c]:Number of samples*

**(b)**

| **Carotenoids content (mg 100 $g^{-1}$ dry sample), v=3[a]** | **Optimized UAE extract** | **Optimized MAE extract** | **Folch extract** |
|---|---|---|---|
| Astaxanthin | 3.64(±0.29) | 5.79(±0.53) | 1.61(±0.54) |
| Canthaxanthin | 2.13(±0.64) | 1.39(±0.36) | 0.39(±0.26) |
| Zeaxanthin | 0.64(±0.34) | 2.53(±0.42) | 0.84(±0.25) |
| Lutein | 0.23(±0.31) | 2.61(±0.13) | 0.69(±0.34) |
| **Final carotenoids content expressed in mg 100 $g^{-1}$ of raw shrimp body sample (N=3)[b]** | | | |
| **Average weight (g) of raw shrimp body samples (±stdev), n=10[c]** | 23.4(±2.6) | | |
| **Average weight (g) of lyophilized shrimp body samples (±stdev), n=10[c]** | 4.84(±1.4) | | |
| **Average (%) moisture of raw shrimp body, n=10 [c]** | 79.30 | | |
| | **UAE extracts** | **MAE extracts** | **Folch extract** |
| **Astaxanthin content (mg 100 $g^{-1}$ raw shrimp body) (±stdev)** | 0.75(±0.29) | 1.20(±0.53) | 0.33(±0.54) |
| **Canthaxanthin content (mg 100 $g^{-1}$ raw shrimp body) (±stdev)** | 0.44(±0.64) | 0.29(±0.36) | 0.081(±0.26) |
| **Zeaxanthin content (mg 100 $g^{-1}$ raw shrimp body) (±stdev)** | 0.13(±0.34) | 0.52(±0.42) | 0.17(±0.25) |
| **Lutein content (mg 100 $g^{-1}$ raw shrimp body) (±stdev)** | 0.048(±0.31) | 0.54(±0.13) | 0.14(±0.34) |

*[a]:Number of LC-MS/MS replicates; [b]:Number of extraction replicates; [c]:Number of samples*

Tables 7.4a-b summarize the results of UAE and MAE of carotenoids from head and body of *A. antennatus* shrimp. From the extraction results, it was conducted that shrimp head contains higher carotenoid content than the body, an evidence that is also supported by the literature [54, 194]. Due to the short optimal extraction times (≤10 min) in both extraction methods, the carotenoids extraction yields are probably ascribed to the high value of US power and to the higher temperature of MAE process. In all cases, astaxanthin was extracted in higher amounts compared to the other studied carotenoids.

In the case of shrimp head, the extraction yields of UAE and MAE did not present any statistically significant difference ($p$-value≥0.05). Shrimp head is an important source of caroteno-proteins, which are complexes of carotenoids with high density lipoproteins [238]. Carotenoproteins can be divided in two different types. When the carotenoid is stoichiometrically bound to a simple protein or a glycoprotein, the complex is classified as true carotenoprotein. The structure of a carotenoid associated with a lipo(glycol) protein is characterized as carotenolipo(glycol) protein or lipovitellin [239]. Carotenoids could be released from the carotenoprotein complex with the use of organic solvents, like acetone or possibly by the implementation of high energy. In addition to the use of acetone as extraction solvent, the conditions applied for UAE or MAE of carotenoids, in this study, could possibly result to the destruction of the carotenoprotein structure and therefore to the release of the free form of carotenoids bound to the protein complex providing this way significant (p-value<0.05) higher content of astaxanthin from the head compared to the body.

On the other hand, MAE prevailed in terms of carotenoid yields in shrimp body. Concerning the extraction yield of shrimp body carotenoids, MAE seemed to reveal 2-times higher content of carotenoids than UAE. The lower content of carotenoids in UAE could be attributed to xanthophylls (mainly zeaxanthin and lutein) degradation caused by the more pronounced effect of high US power [228] compared to that provoked by MAE temperature. This hypothesis is enhanced by the fact that zeaxanthin and lutein content were higher in MAE compared to UAE (Table 7.4b).

In the present study, UAE and MAE of shrimp carotenoids resulted in higher extraction yields compared to conventional techniques [240, 241]. According to

previous studies [153, 241], only supercritical $CO_2$ extraction has shown higher extraction yields compared to high-energy techniques for shrimp carotenoids. Nonetheless, UAE and MAE are simpler and cost-saving techniques compared to the elaborated instrumentation of supercritical extraction. It is, also possible that a synergistic combination of UAE and MAE techniques could lead to even greater extraction yields of bioactive compounds and therefore, improve the efficacy of high-energy extraction procedures compared to that of conventional methods [121].

To conclude, even though UAE and MAE were applied to same matrix, the different body components of shrimp (head and body) required different techniques in order to extract the maximum content of carotenoids, especially astaxanthin. **UAE** would be preferred in **shrimp head** since it was faster and **MAE** would be the right choice in **shrimp body** since it extracted almost 2-times more carotenoids.

# CHAPTER 8
# EVALUATION OF THE EFFECT OF EXTRACTION METHODS AND EXTRACTION PARAMETERS ON THE METABOLIC PROFILE OF APRICOT DOE-EXTRACTS USING NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

## 8.1 Nuclear magnetic resonance (NMR) spectroscopy and metabolomics

Nuclear magnetic resonance (NMR) has proven an invaluable tool in plant science since it is a robust, quick, reproducible, non-destructive and relatively easy to use analytical platform that does not require laborious sample preparation and can simultaneously identify diverse groups of compounds. NMR metabolomics focuses primarily on secondary metabolites and has proven its ability to differentiate plants from different origin, or even to highlight variations after different treatments or processes. It has been also used to study the plant metabolite composition influenced by extraction methods, sample collection seasons and drying methods in order to predict the activity and toxicity of plant extracts, absolute prerequisite in drug development. Metabolomic analysis generates huge datasets that render necessary the application of chemometric methods. Lately, the metabolomics strategy based on NMR spectroscopy has been employed as a new analytical method for the stricter standardization, quality control and authentication of phytomedicines [171].

It is well accepted that the quality of information obtained from a metabolomic study critically depends on sample pretreatment including extraction. Only comprehensive and reproducible extraction techniques and methodologies will provide reliable data in terms of the metabolome present in the samples for analysis. To-date, there is only scarce data regarding the implementation of validated high energy extraction methods to the field of food-metabolomics.

As it was already mentioned above, experimental design (DOE) improves the assets of extraction methods through the accurate optimization of all extraction

parameters by running a smaller necessary number of experiments. In addition, DOE models answer the questions of a) ‘which parameters are crucial for the extraction?’, b) ‘do the interactions between extraction parameters affect critically extraction yields?’ and c) ‘is it possible to enrich the obtained extract with different co-extracting phytonutrients from the substrate by modifying and adjusting the extraction conditions?’. The last topic is of unique importance for the industry of natural products since even when an extraction is selective, the final natural extract also contains other secondary metabolites in lower concentrations.

Regarding the former issue, $^{1}$H-NMR provides invaluable data through the assessment and metabolic characterization of UAE- and MAE extracts [171]. Multivariate chemometric analysis of NMR datasets unveil the metabolites variations in high-energy extracts and differentiate them based on the extraction conditions [242]. For instance, what solvent polarity do I need to obtain a carotenoid extract containing branched-chain amino acids? Or can I recover a polyphenol extract rich in fatty acids by applying short extraction time and high US or MW energy? As a result, NMR spectroscopy could endorse a future customized production of multi-compounds nutraceutical products extracted from the same natural source.

In relation to the current project, NMR spectroscopy was implemented as a robust tool in the developed analytical platform for the NMR-based metabolic profile of DOE apricot pulp extracts under different extraction conditions and the generation of multivariate statistical models (both unsupervised and supervised) for the classification of high energy extracts by elucidating the key metabolites responsible for extracts’ discrimination.

## 8.2 NMR based metabolic profiling for DOE apricots extracts

Nuclear magnetic resonance (NMR) is one of the most implemented and efficient analytical platform used for the elucidation of metabolites from complex mixtures, including natural extracts. Due to the high significance of apricots for the Greek economy and the prompt need for the future valorization of apricot byproducts, NMR spectroscopy was used as a complementary technique to identify other secondary metabolites of carotenoids extracts (Fig. 8.1).

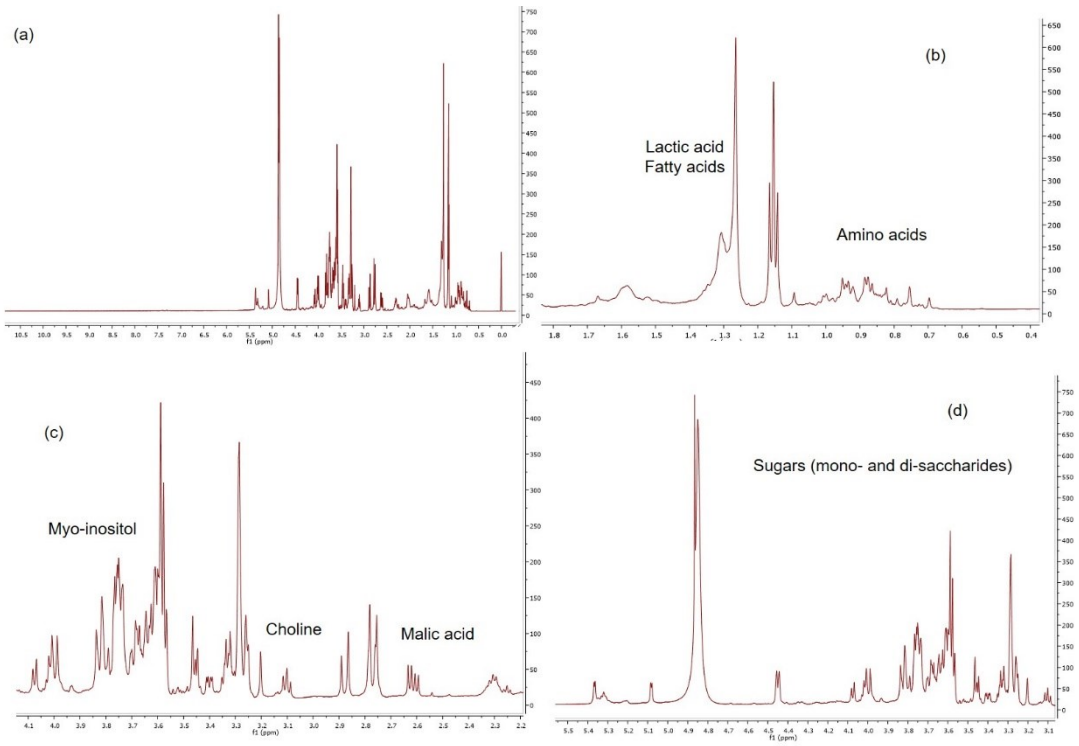



**Figure 8.1. 1D NMR spectrum of a characteristic apricot extract (i.e. UAE_Ethanol-Acetone extract). (a) 1H NOESY spectrum (b) Chemical shifts region of amino acids, lactic acid and fatty acids (c) Chemical shifts region of myo-inositol, choline and malic acid (d) Chemical shifts region of sugars.**

In Table 8.1 is presented the identification and the characteristic 1H chemical shifts regions of major compounds present in apricot samples.

**Table 8.1 Characteristic 1H NMR peaks of apricot byproducts extracts identified in the PCA groups.**

| Compounds | 1H chemical shift | Peak multiplicity |
|---|---|---|
| Valine | 0.99, 1.04, 2.28 | (d), (d), (m) |
| Leucine | 0.98, 0.96 | (d, J=7.5), (d, J=7.5) |
| Isoleucine | 0.94, 1.01, 1.25, 1.45, 1.96, 3.66 | (t), (d), (m), (m), (m), (m) |
| Alanine | 1.48 | (d) |
| Lysine | 1.61 | (t) |
| Choline | 3.10 | (s) |
| Fatty acids | 1.26 | (m) |
| Myo-inositol | 3.67, 3.78 | (t), (t) |
| Malic acid | 2.68, 2.78 | (dd), (dd) |
| Lactic acid | 1.34 | (d) |
| Formic acid | 8.40 | (s) |
| Fructose | 3.53, 4.04 | (t), (t) |
| Sucrose | 4.18, 5.39 | (d), (d, J=3.9) |
| Glucose | 5.12 | (d) |
| Xylose | 5.07 | (d) |

(s): single peak, (d): double peak, (dd): two double peaks, (t): triplet peak, (m): multiple peak, J: coupling constant

The first part of NMR analysis copes with how the extraction parameters (extraction techniques, extraction solvent and time, US/MW power and solvent/material ratio) shape the profile of co-extracted metabolites. Particularly, the samples that were examined were (i) the extracts of all different

UAE and MAE solvents, (ii) DOE-extracts of lower and higher carotenoids content of high energy extraction methods and (iii) Folch extracts.

Principal component analysis (PCA) was applied to provide a general and unbiased overview of the trends and possible outliers of high energy extracts [242]. The clusters provided by PCA were then color-coded in order to facilitate the processing and interpretation of the ensued models. A complementary LC-MS/MS analysis (Supplementary data, Table S14) of the NMR extracts affirmed that PCA grouping was also hinged on final carotenoids content. Based on that remark, extracts with relatively low carotenoid content (under 5 mg 100 $g^{-1}$ dry sample) formed Group 1 (green dots). Group 2 (blue dots) contained those with medium carotenoid content (between 5-15 mg 100 $g^{-1}$ dry sample) and Group 3 (red dots) consisted of extracts with high carotenoids yield (over 15 mg 100 $g^{-1}$ dry sample) (Table 8.2).

**Table 8.2. Apricot pulp samples classification produced by PCA model.**

| Low extraction yield (≤5 mg carotenoids/100 g dry sample) | Medium extraction yield (5-15 mg carotenoids/100 g dry sample) | High extraction yield (≥15 mg carotenoids/100 g dry sample) |
|---|---|---|
| MAE_$CHCl_3$-MeOH_2:1 | MAE_$CHCl_3$-MeOH_1:1 | MAE_Run 2_Two level design |
| MAE_CHCl3 | MAE_EtOH-Acetone | MAE_Run 3_Two level design |
| MAE_Acetone | MAE_EtOH | MAE_Run 8_Two level design |
| MAE_*n*-Hexane-Acetone | UAE_EtOH-Acetone | MAE_Run 6_BBD |
| MAE_*n*-Hexane-Acetone-EtOH 2:1:1 | UAE_EtOH | MAE_Run 9_BBD |
| UAE_$CHCl_3$-MeOH_2:1 | UAE_$CHCl_3$-MeOH_1:1 | MAE_Run 12_BBD |
| UAE_$CHCl_3$ | UAE_Run 3_Two level design | UAE_Run 4_Two level design |
| UAE_Acetone | UAE_Run 7_Two level design | UAE_Run 8_Two level design |
| UAE_*n*-Hexane-Acetone | UAE_Run 13_BBD | MAE_Optimal values |
| UAE_*n*-Hexane-Acetone-EtOH 2:1:1 | UAE_Optimal values | |
| UAE_Run 6_BBD | | |
| UAE_Run 11_BBD | | |
| Folch | | |

PCA clustering revealed that the primary differentiating factor for sample classification was the **polarity** of extraction solvent rather than the extraction technique used (Fig. 8.2). The effect of extraction method was outlined implicitly since every solvent shows a different behavior when interacts with US or MW due to its different physical properties (polarity, viscosity, vapor pressure, diffusion coefficient etc.).

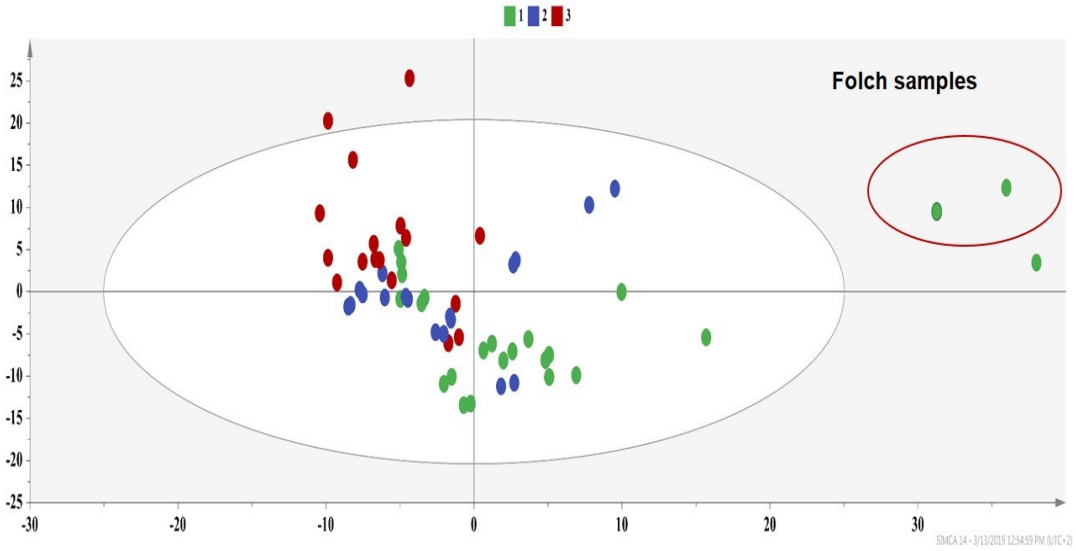



**Figure 8.2. DOE-extracts classification from initial PCA model. PCA-X, A=2, N=57 $R^2X(cum)=0.73$, $Q^2(cum)= 0.57$, Green dots= Apricot pulp samples with low extraction yield (≤5 mg carotenoids/100 g dry sample) Blue dots= Apricot pulp samples with medium extraction yield (5-15 mg carotenoids/100 g dry sample) Red dots= Apricot pulp samples with high extraction yield (≥15 mg carotenoids/100 g dry sample)**

In the initial PCA model, Folch samples were outliers, a fact which supports the LC-MS/MS results (Table 7.3), where classic methods presented the lower carotenoids yields, proving this way that the conventional techniques exhibit a distinct metabolic profile compared to high energy approaches. These samples contained extremely high content in valine, isoleucine and leucine, fatty acids, lactic acid, alanine and malic acid as presented in the contribution plot in Figure 8.3.

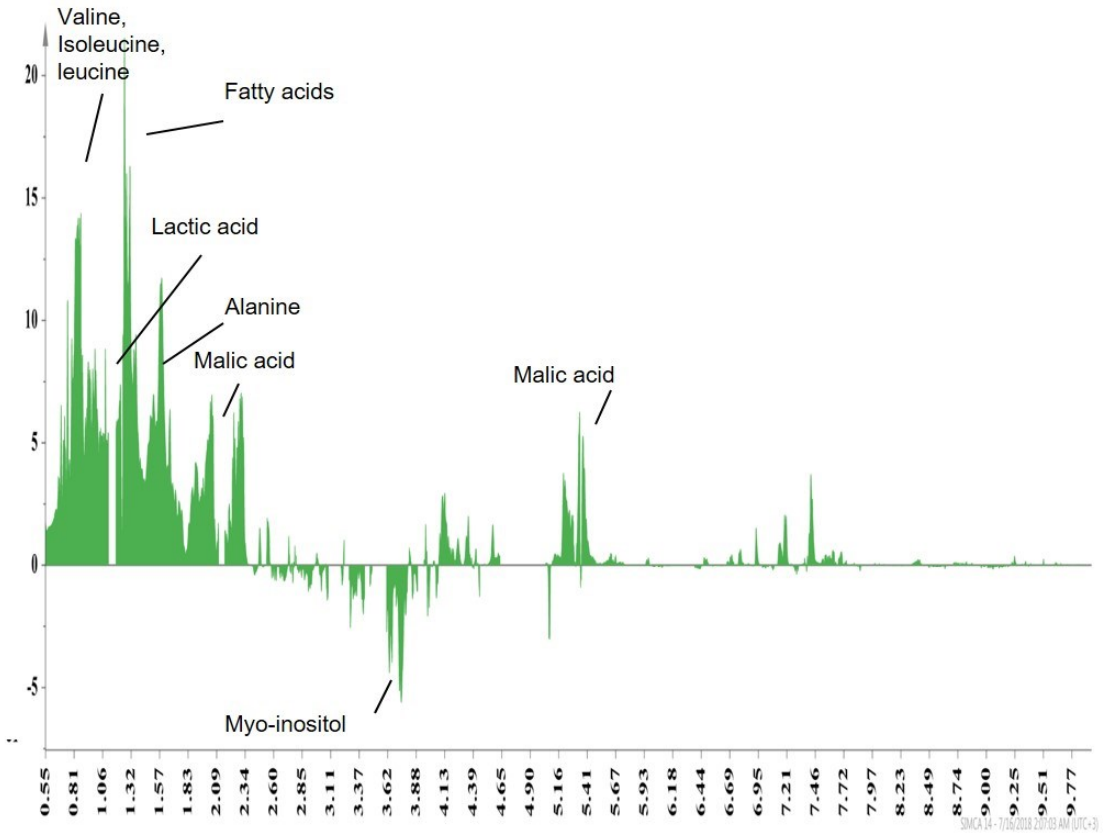



**Figure 8.3. Contribution plot of Folch samples.**

Since the three groups were differentiated along the first principal component (PC1), exclusion of Folch samples produced the final PCA model (Fig. 8.4a). Besides solvent polarity, the grouping of extracts in different clusters gave a hint for the effect of extraction parameters, highlighting as more critical variables the US/MW power and secondly the solvent/material ratio (Fig. 8.4a). In fact, the localization of each group along the first principal component postulates a quantitative trend, noticeably Group 1 mainly consists of the lowest concentration in fatty acids and monosacharides. Moving to Group 2 which is characterized by high content in myo-inositol and branched chained amino acids while the highest in fatty acids. Finally, the Group 3 contains samples with the highest levels in mono-, di- saccharides, and branched chained amino acids.

The metabolites with high discriminant power, which are responsible for the grouping of the apricot extracts' samples were revealed by the corresponding loading plot (8.4b). According to this plot, the samples of Group 1, are characterized from metabolites with negative first component (p1) and positive

second component (p2), principally branched amino acids (valine, leucine, isoleucine), as they are scattered in the second and third quadrant. Based on Figure 8.4a, Group 2 and 3 were clearly discriminated from Group 1. The extracts of these two groups are mainly located in the fourth quadrant, which are described by a positive first component (p1) and a negative second component (p2). The metabolites that are significant for this classification according to loading plot (Fig. 8.4b) were myo-inositol, fatty acids, mono- and disaccharides.

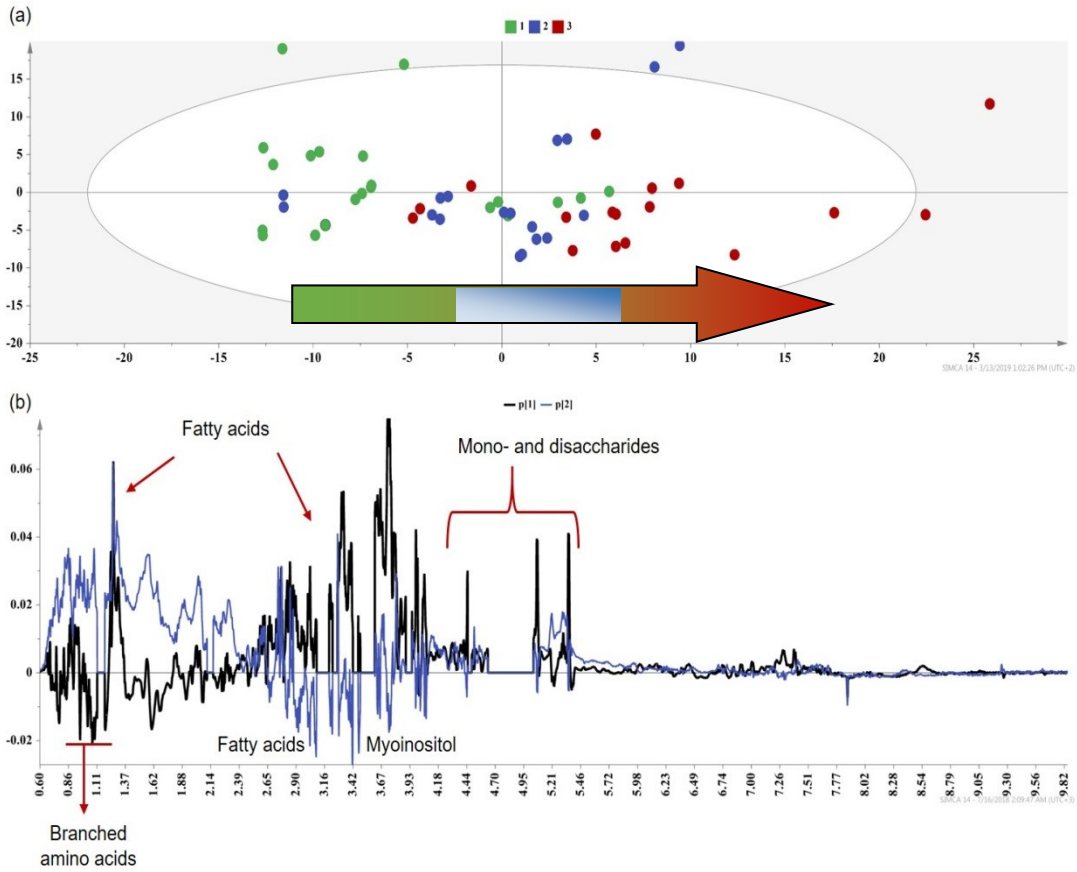



**Figure 8.4. (a) DOE-extracts classification from final PCA model PCA-X, A=2, N=54 $R^2X(cum)=0.87$, $Q^2(cum)= 0.67$, Green dots= Apricot pulp samples with low extraction yield (≤5 mg carotenoids/100 g dry sample) Blue dots= Apricot pulp samples with medium extraction yield (5-15 mg carotenoids/100 g dry sample) Red dots= Apricot pulp samples with high extraction yield (≥15 mg carotenoids/100 g dry sample) (b) loading plot of the final PCA model.**

## 8.3 Supervised statistical models using Orthogonal partial least square-discriminant analysis (OPLS-DA)

Subsequently, class information was embedded into supervised models of orthogonal partial least square-discriminant analysis (OPLS-DA) to identify the key metabolites responsible for extracts differentiation [171]. Contrary to *vis*-spectrophotometric and LC-MS/MS assays, NMR focuses on the simultaneous elucidation of co-extracted metabolites other than carotenoids that shape the different metabolic profiles of each group. According to their $R^2Y(cum)$ and $Q^2(cum)$ values, the generated OPLS-DA models provided a valid separation of DOE-extracts (Fig. 8.5a-8.8a). In each case, the discriminant co-extractives were highlighted by S-line plots (Fig. 8.5b-8.8b) and identified by processing 2D NMR spectra (gCOSY, gHMBC, gHSQC) (Supplementary data, Figures S14-16).

S-line plot depicts and compares the actual 1H-NMR spectra of each group. Separated by first principal component, samples that belong on the first and fourth quadrant are represented by the spectrum which is on the down side of the x-axis, while samples that belong on the second and third quadrant are represented by the spectrum on the up side of the x-axis. The discriminant power of each metabolites is demonstrated with a color code and shows an increasing trend from green to red. Thus, the peaks of the metabolites which are important for the clusters discrimination are depicted with orange to red color.

Notably, the secondary metabolites with high discriminant power were branched-chain non polar amino acids (valine, leucine, isoleucine) and basic amino acid lysine, myo-inositol, fatty acids, choline, monosaccharides (fructose, glucose, xylose) and disaccharides (sucrose). The respective concentration trends of each metabolite responsible for the discrimination is framed in box plots (Fig. 8.6c-8.8c), disclosing a pattern in line with carotenoids yield and UAE/MAE parameters. In total 15 metabolites were identified. Validation steps, including permutation testing and receiver operating characteristic (ROC) curves, were employed and verified OPLS-DA models reliability (Supplementary data, Figures S17a-d).

In particular, the class information enclosed in the first OPLS-DA (Fig. 8.5a) model included the group with low carotenoid content (Group 1) and one group comprised of Group 2 and Group 3 due to the vicinity of these two groups in Figure 8.4a PCA model.

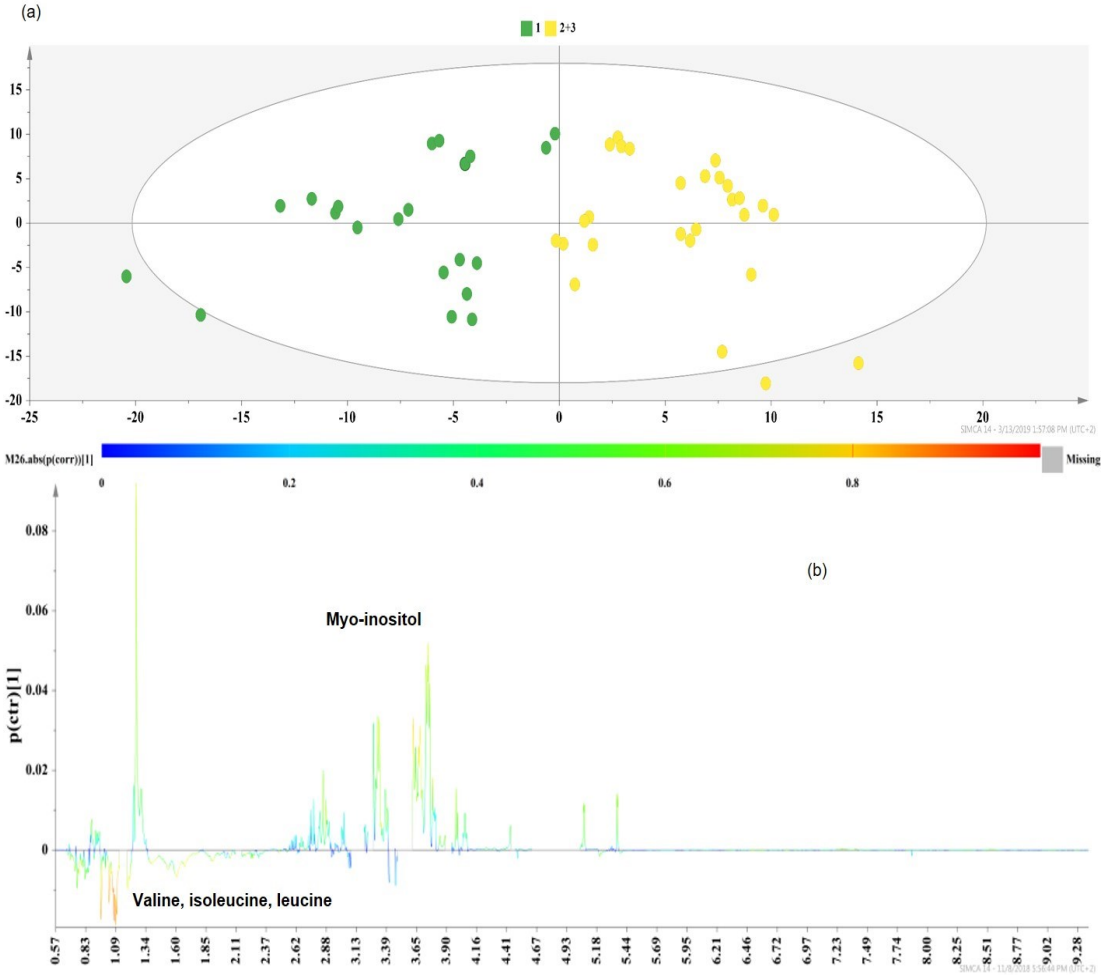



**Figure 8.5. (a) OPLS-DA model of Group 1 vs Group 2+3 DOE apricots extracts, Pareto scaled, A=1+1+0, N=48, R2Y(cum)=0.706, Q2(cum)=0.596 (b) S-line plot of the OPLS-DA model.**

Extracts of low carotenoid yields were enriched in branched-chained hydrophobic amino acids (valine, leucine and isoleucine) (Fig. 8.5b). The above mentioned amino acids are among apricots' beneficial secondary components due to their numerous biological activities [243]. The co-extraction of these metabolites was favored at more non polar solvents (acetone, chloroform, *n*-hexane) or their mixtures with methanol. According to published studies [244]. UAE conditions, especially time and US power, could initiate amino acids degradation reactions in cases where highly polar solvents are used. Thus, the

combination of less polar solvents with relatively low US power (575-625 W) seems to provide apricots pulp extracts with important levels of certain amino acids [244, 245] (Fig. 8.5b).

An additional marker affecting the clear discrimination of Group 1 (low carotenoids content) from the cluster of Groups 2 and 3 (higher carotenoids yield) along the second principal component (PC2) is the high content of myo-inositol in the latter groups (Fig. 8.5b). Myo-inositol is a sugar polyalcohol which participates in plants development by promoting the biosynthesis of molecules responsible for cell wall structure [246, 247]. Our results were in agreement with other works where cyclitols (myo-inositol, d-pinitol, etc.) were extracted mostly at higher UAE solvent volumes and extraction times [248]. As stated by Ruiz-Aceituno et al (2016) [249], MAE extracts containing inositols were obtained at short extraction times and high solvent/material ratios, a finding that was also depicted in Figures 8.5a and b [249].

To a step further, an **in-pairs** OPLS-DA investigation of the three carotenoid-dependent groups pinpointed additional differentiating metabolites. Apart from branched-chain amino acids and myo-inositol, other prevalent components in the separation of Group 1 (low carotenoid yields) and Group 2 (carotenoid yields between 5-15 mg 100 $g^{-1}$ dry sample) along the second principal component (PC2) were choline and fatty acids, which exhibited higher values in Group 2 (Fig. 8.6a and b). In more details, box-plots of this OPLS-DA model (Fig. 8.6c), showed that in Group 1 valine and leucine content was 3-times higher than in Group 2. On the other hand, choline and myo-inositol were 2-fold and fatty acids content 4-fold higher in Group 2.

Choline is acknowledged as an essential macronutrient, mainly present in lipid foods or plant oils (i.e. apricot kernel oil) mainly as choline derivatives with lipid components (glycerophosphocholine, phosphocholine, sphingomyelin) [250]. Moreover, oleic (C18:1) and linoleic acids (C18:2) are two of the most predominant fatty acids in lipid fraction of apricot byproducts' [251], with content of ~20% and ~10% of the total unsaturated fatty acids content, respectively. As shown in Figure 8.6b, extracts of nonpolar solvents and UAE extracts of relatively high extraction times (20-30 minutes) and US power (625-675 W) presented increased levels of choline and fatty acids. Specifically, fatty acids

concentration differences and variations between the groups were determined by using the characteristic peak at 1.30 ppm, which correspond to the overlap of methylene $–CH_2-$ (except of those in position α- and β- from the carboxyl group) of the fatty acids chain. This outcome is consistent with the results of Hernández-Santos (2016) [252] concerning the effect of UAE parameters on fatty acid profile of pumpkin lipid fraction [252].

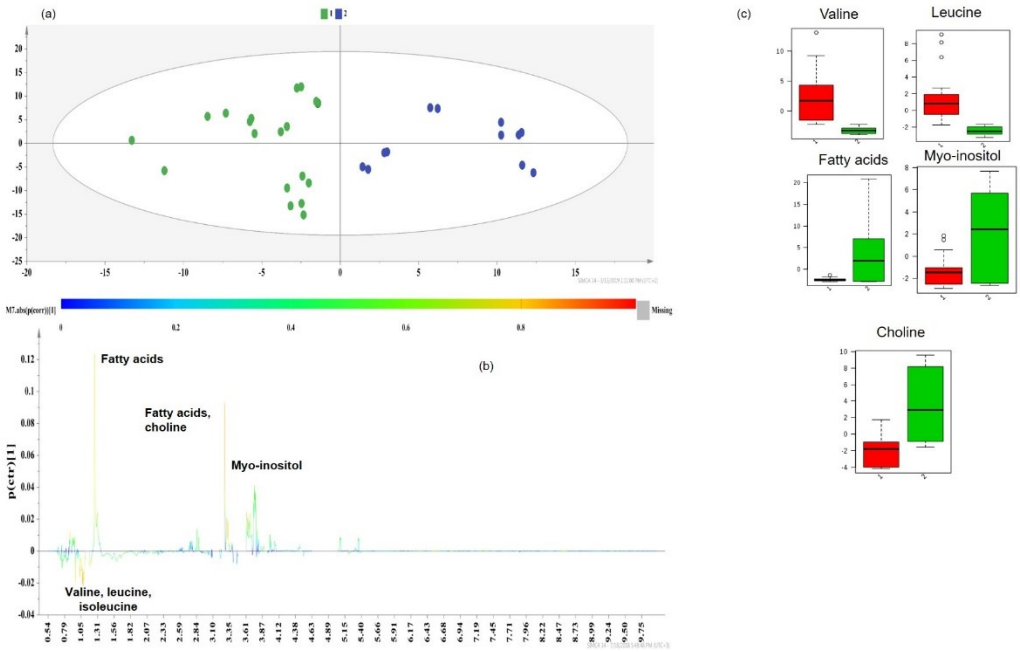



**Figure 8.6. (a) OPLS-DA model of Group 1 vs Group 2 DOE apricots extracts, Pareto scaled, A=1+1+0, N=31, $R^2Y(cum)=0.725$, $Q^2(cum)=0.630$ (b) S-line plot of the OPLS-DA model (c) box-plots of the discriminant metabolites.**

Following a similar pattern, the discrimination of Group 1 and Group 3 (Fig. 8.7a) was attributed to the same metabolites (Fig. 8.7b). Valine, isoleucine and leucine concentration was from 2-times (valine) to almost 4-times higher (leucine) in Group 1, while isoleucine content was near 5-times more also according to box-plots (Fig. 8.7c). Furthermore, Group 1 presented 3-times higher content of lactic acid, which is present in unripe apricots and fruit byproducts [253]. However it is interesting to note that the role of myo-inositol and fatty acids was equally significant for this classification compared the previous OPLS-DA model (i.e. Group 1 vs Group 2). This observation could be ascribed to the presence of MAE extracts in Group 3. As reported by Esquivel-Hernández (2017) [254], MAE recovery of lipids is more efficient in higher extraction times than the ones of the studied extracts [254]. In addition, mono-

saccharide fructose content was 2.5-fold higher in Group 3, a fact that could be attributed to the higher temperatures and solvent/material ratio of MAE extracts and higher US power of UAE extracts of Group 3 [255-258].

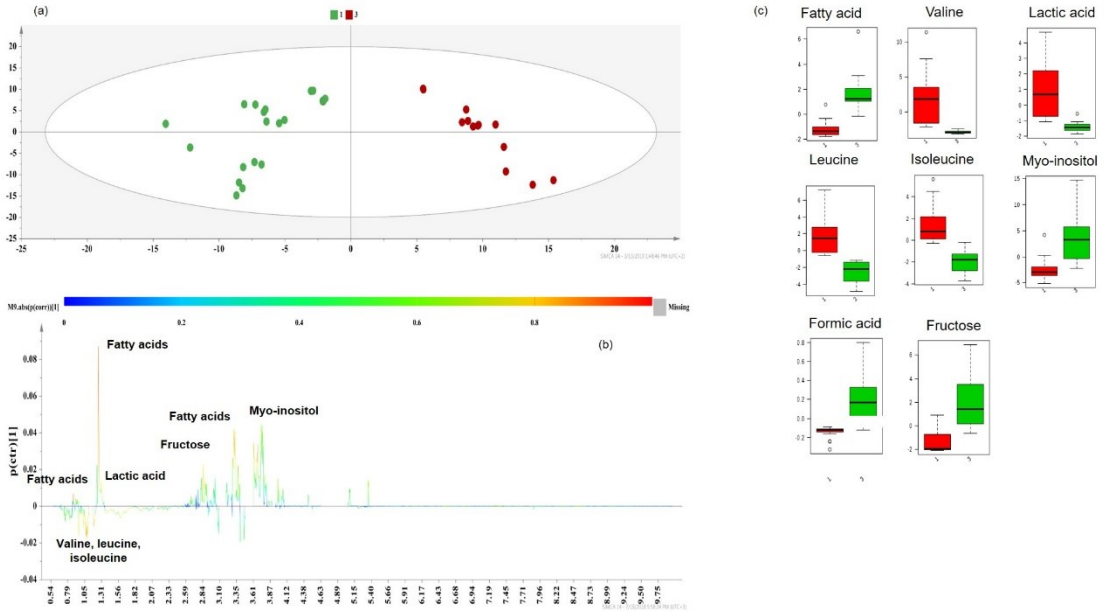



**Figure 8.7. (a) OPLS-DA model of Group 1 vs Group 3 DOE apricots extracts, Pareto scaled, A=1+1+0, N=32, $R^2Y(cum)=0.889$, $Q^2(cum)=0.836$ (b) S-line plot of the OPLS-DA model (c) box-plots of the discriminant metabolites.**

Ultimately, the trend along the second principal component (PC2) resulted in the discrimination of DOE-extracts with medium (Group 2) and high (Group 3) carotenoids content (Fig. 8.8a). According to box-plots (Fig. 8.8c), Group 2 contains amino acids in lower amounts than the Group 1, but valine, leucine and lysine were metabolites responsible for the discrimination of Group 2 and Group 3, with a 4-, 1.5- and 2.5-fold higher content, respectively. However, the major metabolites playing a crucial role on the separation the two groups were apricots' mono- and disaccharides [255, 256] (Fig. 8.8b). Group 3 includes primarily MAE extracts. Higher extraction temperatures of MAE (~60 ºC) and solvent/material ratios facilitate the extraction of sugars, like glucose, xylose and fructose [257]. High US powers of UAE extracts in Group 3 also promote monosaccharides (glucose, xylose) recovery [258].

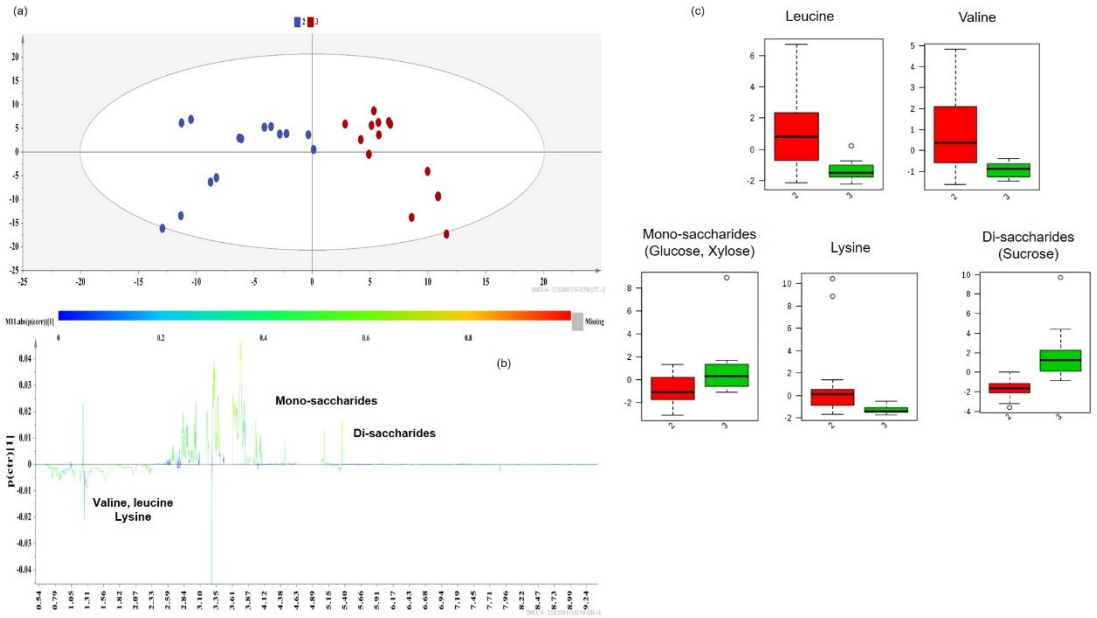



**Figure 8.8. (a) OPLS-DA model of Group 2 vs Group 3 DOE apricots extracts, Pareto scaled, A=1+1, N=27, $R^2Y(cum)=0.780$, $Q^2(cum)=0.642$ (b) S-line plot of the OPLS-DA model (c) box-plots of the discriminant metabolites.**

The variations in the metabolic map of apricot extracts could be annotated in Figure 8.9, where characteristic spectra from each group were superimposed to reveal their discriminant metabolites. In details, the superimposed spectra belonged to (a) optimal UAE (blue spectrum) (b) optimal MAE (red spectrum) and (c) Folch apricot pulp extracts. Folch extracts, which are included in Group 1, presented peaks in amino acids region since they had the higher content of leucine, valine and isoleucine. On the other hand, optimal UAE (Group 2) and optimal MAE extracts (Group 3) contained, according to Figure 8.9, higher contents of fatty acids, myo-inositol and sugars.

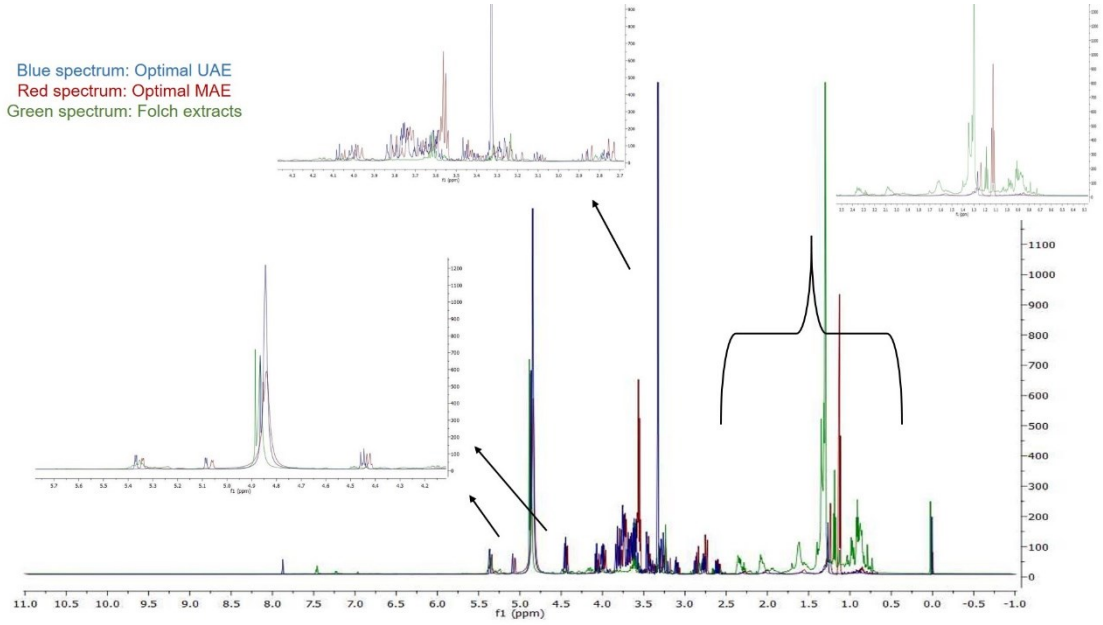



**Figure 8.9. Superimposed spectrum of (a) optimal UAE (b) optimal MAE and (c) Folch extracts.**

To conclude, a final extract of **lower carotenoid content and branched-chain amino acids** could be obtained by using **non-polar solvents in UAE or MAE**. A product containing **choline, unsaturated fatty acids, sugar alcohols and medium carotenoids content** would be delivered either at **high US power and times** or **MAE short extraction time and higher solvent volumes**. Finally, a **sugar- and carotenoid-rich extract** was provided when **MAE at high MW power, higher temperatures compared to UAE and higher solvent volumes** was applied.

In conclusion, the NMR-based screening of extracts, delivered by different extraction methods and by distinct extraction conditions, should be considered as a quite useful basic research tool for enabling the standardization and for directing a future large-scale extraction procedure towards the acquisition of extracts containing different bioactive components with 'tailor-made' – according to the case- biological activities.

Taking as an example the future valorization and the prospect of transforming a byproduct to high-added value products, the potentials of NMR spectroscopy on apricots byproducts were explored.

Thus:

a) When a **high-carotenoid content** extract, **clean** from other co-extractives, will be the final product of an industry-scale extraction process, **UAE with high US power (Group 2)** should be preferred. Despite the fact, that the extracts of Group 3 (mostly MAE extracts in higher temperature, time, MW power and solvent/material ratio) provided higher carotenoid yields, the main co-extractives of these samples were **sugars**, whose removal and clean-up could be more challenging and laborious than that of other co-extractives.

b) Furthermore, extracts of Group 2, either these obtained by UAE at high US power or those acquired with MAE at short extraction times, could be resulted in an extract with combined biological activities due to its significant content in carotenoids (improving eye health, immune-modulating properties, anti-allergic, anti-aging, sun-protective activity), choline (enhancing brain health and cognitive function, affecting detoxification pathways and organs, like liver and kidneys), myo-inositol (promoting female fertility, treating polycystic ovary syndrome, reducing anxiety, restoring insulin resistance) and unsaturated fatty acids (health promoting agents against fat burning, affecting inflammation, steroid signaling and membrane-bound proteins function, participating in glucose/lipid metabolism).

Therefore, these results should be considered as an elementary index or a starting point for the development of nutraceutical supplements. However, further investigation is required in order to determine the exact concentration of these components and the comprehensive biological activities of the final extracts.

# CHAPTER 9
# CONCLUSIONS AND FUTURE PERSPECTIVES

## 9.1 Conclusions

In recent years, coping with the problem of agro-byproducts and accumulation and disposal created new potentials for the profitable and eco-friendly management of food and agro-waste. Introduction of alternative uses of agro-products through their transformation to valuable nutraceutical and cosmetics ingredients looms as a sustainable approach in terms of industry and economy. Thus, the valorization of apricot by-products could be a real shot in the arm for Greek agro-oriented market taking into account the fact that apricots are one of the most merchantable local commodities.

Furthermore, egg yolk is one of the most important dietary sources of bioactive carotenoids, which act as enhancers of ocular health. Due to their lipophilicity, lutein and zeaxanthin could be consumed and accumulated more effectively in human body when a lipid matrix, like egg, is the carrier. On the other hand, the lipidic matrix of egg yolk forms complex structures where carotenoids are bound mainly with hydrophobic interactions. Therefore, egg yolk is the ideal example of lipid matrix in order to develop and validate a new analytical procedure for the study and recovery of egg yolk xanthophylls.

Moreover, the recovery of a high-added value group of bioactive molecules (carotenoids) from marine organisms, such as shrimps, can constitute a profitable and valuable commercial alternative, as these compounds can be used as dietary supplements, food color enhancers and additives in animal feeds, functional foods, preservatives, pharmaceuticals and cosmetics.

In the current project, the results of the implementation of UAE and MAE for the recovery of carotenoids from natural sources are summarized below:

- Ultrasound- (UAE) and microwave-assisted extraction (MAE) of bioactive compounds from natural substrates is a **matrix-dependent** procedure since the optimal extraction conditions for each substrate were different.

- **Apricot byproducts'** carotenoids were obtained in higher amounts when **MAE** was applied. **UAE** should be considered as the most adequate extraction tool compared to MAE and the conventional Folch for recovering carotenoids from complex lipid substrates, like **egg yolk**. Since UAE and MAE provided similar results in the extraction of astaxanthin from **shrimp head**, **UAE** was preferred due to the shorter extraction period and due to the possible release of carotenoids from carotenoproteins provoked by UAE extraction conditions. In the case of **shrimp body**, **MAE** was more adequate for the extraction of carotenoids.
- Among all tested extraction parameters, **extraction solvent**, **extraction time** and **US or MW power** affected more the final carotenoid content.
- Compared to classic extraction (Folch method), high energy extractions provided in all cases, **higher** (even **19-times more)** carotenoid yields.
- High energy extraction methods achieved higher carotenoid yields at extraction times **lower of 30 minutes**.
- The application of US and MW power could provoke the **isomerization** of *trans*-carotenoids forms to their *cis*-isomers, thus special attention should be paid for the selection of the appropriate extraction conditions.
- The substitution of conventional organic solvents with green ecofriendly natural deep eutectic solvents (NADES) resulted in even **4-times higher carotenoid yields** when US or MW irradiation was not very short (**over 10 minutes**). In general, the **increased temperatures of MAE** method enhance the outcome of carotenoid extraction.
- The utilization of **experimental design (DOE)** models decreased the number of experimental runs and provided an accurate, fast and reliable optimization of high energy extraction methods.
- The developed LC-MS/MS method quantified the target carotenoids in short analysis time even at relatively low carotenoids concentrations.
- Nuclear magnetic resonance (NMR) spectroscopy combined with multivariate statistical analysis models (PCA, OPLS-DA) revealed the metabolic fingerprint of apricot extracts and designated the extraction

conditions that must be applied in order to obtain carotenoid extracts with different groups of co-extractives.

- **Non-polar UAE or MAE solvents** provided extracts of **lower carotenoid content and branched-chain amino acids** (valine, leucine, isoleucine).
- **High US power and times** or **MAE short extraction time and higher solvent volumes** extracted **choline, unsaturated fatty acids** (oleic acid, linoleic acid)**, sugar alcohols** (myo-inositol) **and medium carotenoids content.**
- **MAE at high MW power and solvent volumes along with higher MAE temperatures** resulted in **sugar- (glucose, xylose, fructose) and carotenoid-rich extracts.**
- In the case of a prospective scaling-up of high energy extraction processes in the field of nutraceuticals or cosmetics, **UAE** probably emerge as a more **suitable** approach, as it delivered extracts with significant carotenoid content, rich in other bioactive constituents (choline, myo-inositol, fatty acids) and lower sugar concentration compared to MAE extracts.

Thus, summarizing the results of this research, high energy extraction methods emerge as an attractive alternative from industrial point of view, as they provide high quality extracts.

In particular, optimizing the efficiency and quality of extraction procedures is a significant milestone in setting out new strategies for the recovery of natural extracts with health-promoting properties. High energy techniques emerge as a viable solution suitable for tackling any challenges or drawbacks of the current large-scale extraction methods.

To date, the first study is the first that hyphenates innovative high energy extraction techniques, DOE models and APCI(+) LC-MS/MS and NMR spectroscopy to offer a robust analytical tool for extracting carotenoids from natural byproducts and lipid matrices. Furthermore, the integration of high energy practices with high-throughput analytical tools (LC-MS/MS, NMR) generate a robust platform able to provide various bioactive fractions according the customized needs of different industrial sectors (pharmaceutical,

cosmetics, nutraceuticals, food, etc.). Concerning the current project, correlating extracts of different carotenoids yields with particular secondary metabolites by adjusting UAE or MAE parameters allows the imminent standardization of the extraction procedure to obtain multi-targeted and multi-functional natural extracts. In that way, high energy extractions are re-evaluated and upgraded to reliable sample preparation steps in the field of metabolomics.

In addition, NADES experimental design-based UAE and MAE stand out as an innovative green alternative to traditional extraction processes for the revalorization of food waste and agro-industry byproducts. In this work, we managed to propose a simple, efficient and versatile procedure for the extraction of valuable non-polar compounds from apricots pulp and shrimp head.

Regarding b-carotene extraction from apricots pulp, NADES-UAE and MAE reached to notably increased extraction efficiency compared to other conventional extraction methodologies. NADESs could also be considered as a future solvent for the extraction of astaxanthin from lipid substrates, like shrimps, since it provided similar extraction yields to organic solvent systems when MAE was implemented. Based on our results, MAE should be preferred over UAE for the recovery of non-polar carotenoids due to the increased temperatures that enables cell disruption and help compounds release. Hence, since NADESs are acknowledged as eco-friendly and non-toxic solvents, CC/TA carotenoid extracts could find wide applications in nutraceutical and cosmetics industry.

In short, the goal of the current study is to deepen the knowledge referring to the future applicability of the described combinatorial analytical platform aiming at the designation and commercialization of agro- or seafood byproducts and lipid foods as high-added value products.

## 9.2 Future perspectives: Large-scale high energy extraction, is it an economically feasible solution?

Pursuing contemporary scientific and commercial needs, high energy systems have already started grabbing the eye of many cosmetic, nutraceutical, pharmaceutical, food and beverage companies, which are focusing their

marketing strategies on natural products and extracts containing bioactive molecules and obtained by high energy extraction technologies.

The winning bet for industrial scale high-energy extraction is its intensification in the production of health-promoting bioactive extracts and in waste management processes for residues of food and drug companies in local and international level. A plethora of economic studies are available in the literature including business plans and feasibility studies for the installation of medium or large scale extraction units. High-energy extraction processes are mostly complex thermodynamic systems with higher capital costs, which are mainly attributed to installation. The engineering design of these systems requires good knowledge of the thermodynamic constraints of solubility and selectivity, and kinetic constraints of mass transfer rate. Modeling of the extraction processes can provide a better understanding of the mechanisms behind extraction and be used to quickly optimize the operating conditions and scale-up any design [78].

Although the economic feasibility analysis for each technique is different, several common general assumptions have to be made including (1) unit working period (hours per day and per year), (2) number of workers per unit extractor and necessary level of expertise, (3) scale-up criteria for each technique like solid feed, solvent flow rate, extraction cell dimensions (i.e., extraction bed for SFE), (4) minimum time to load and unload in each extraction cycle, (5) market price of the extract, (6) matrix initial pretreatment if necessary [259].

Another commonly made assumption is that although in many cases the scale-up from the laboratory to the industrial scale under the same conditions increases the extraction yield, in many studies the yield is kept identical at both scales. Finally, it is necessary to establish the optimal equilibrium between extraction efficiency, extraction duration, physicochemical, and biological properties of the extract and extract quality [259].

Besides the high initial equipment capital required for their industrial-scale implementation, high-energy techniques could result to net profit for the companies because operational expenses and raw material cost, especially

when we refer to natural products waste, are, in many cases, lower compared to conventional methods, like steam distillation. Economic evaluation studies showed that scale-up of extraction units is favored as manufacturing cost is decreasing proportionally (over 50%) when operational units' capacity is enlarging. Increase of extractors' size is translating into more utilities charges (energy consumption, staff payments, maintenance cost), therefore, extraction procedures efficiency in corporate groups normally outcompete those of small-scale businesses [260].

Economic assessment reports of high-energy extraction processes have been increasingly found in literature during the last 5 years [261]. Several factors can attenuate uncertainty in an economic estimation of large-scale high-energy extraction processes. One of the most important sources of uncertainty is the price of the produced extracts (final product), which frequently hinders the correct economic evaluation causing unrealistic estimations.

Focusing on UAE and MAE, the current goal of researchers and ultrasonic equipment companies is the manufacture of pilot US generators and transducers for industrial level adoption and standardization and the design of industrial MW extractors. At this moment, low maintenance cost, final products' enhanced functionality and short amortization period make UAE an economically feasible alternative for obtaining macular carotenoids from complex natural substrates compared to MAE. Nonetheless, MAE systems merit further investigation in order to standardize the operational specifications to ensure high efficiency since until today only MAE prototypes and not continuous commercial MAE systems are launched in the market.

Although the evolution of UAE and MAE systems provides net advantages (short extraction times, high selectivity and extraction yields, wide range of extracted analytes, non-elaborated instrumentation) and these methods are gaining ground concerning their industrial-scale applications, more intense efforts should made in order to comply with any legal limitations or plant unit and safety considerations [83].

## 9.3 Final remarks

The use of emerging technologies in the field of high-energy extraction is spreading widely in many industrial sectors and mainly food, pharmaceuticals, chemicals, and cosmetics. Safety, sustainability, environmental, and economic factors are all forcing laboratories and industry to turn to nonconventional technologies and greener protocols.

The high-energy techniques discussed in the present thesis have certain advantages compared to conventional ones because, in principle, they manage to shorten the extraction time, increase the yield and quality of extracts, and decrease the solvent consumption. However, most of these high-energy extraction techniques are still performing successfully mainly at the laboratory or bench-scale although several industrial applications (medium- or large-scale) can be found mostly as for the case of supercritical fluid extraction (SFE) or accelerated solvent extraction (ASE) [262].

Lately, research groups concentrates upon the enhancement of UAE and MAE through their combination with other technological advances (enzymatic extraction, pressurized liquid extraction, hydro-diffusion and gravity extraction, sono-Soxhlet extraction, sono-clevenger distillation) or green innovative solvents (ionic liquids, natural deep eutectic solvents, non-ionic surfactants at cloud point temperature) for the development of improved hybrid techniques [8].

Presently, the use of new extraction techniques can boost the production and development of functional food, food additives, and ingredients for food and pharmaceutical products. The number of potential applications continues to grow globally, which is reflected to the increase of research articles and patents deposited during the last 5 years. The need for adaptation of these techniques is further supported by the increasing demand of consumers in developed mainly countries toward high-value natural products.

Currently, there is cumulative knowledge in the area, which may provide substantial progress on analytical, engineering, scale-up, and economic issues and may help in its implementation at industrial level. The combination of more comprehensive scale-up works together with the corresponding economic

assessment studies would foster a clearer perception of the techno-economic requirements. Overall, optimized strategies and technological advances in the field of high-energy extraction can provide an economically viable and competitive solution for the preparation and marketing of high-added value natural products for a variety of substrates.

# GLOSSARY TABLE

**Table 1: Glossary table of English to Greek terms**

| English term | Greek term |
|---|---|
| Experimental design | Πειραματικός σχεδιασμός |
| Natural deep eutectic solvents | Φυσικοί βαθέως ευτηκτικοί διαλύτες |
| Liquid chromatography-Mass spectrometry | Υγρή χρωματογραφία-Φασματομετρία μάζας |
| Nuclear magnetic resonance | Πυρηνικός μαγνητικός συντονισμός |
| Ultrasound-assisted extraction | Εκχύλιση με υπερήχους |
| Microwave-assisted extraction | Εκχύλιση με μικροκύματα |
| Response surface methodology | Μεθοδολογία επιφανειών απόκρισης |
| Principal component analysis | Ανάλυση κύριων συνιστωσών |
| Orthogonal partial least square-Discriminant analysis | Διακριτή ανάλυση ορθογώνιας παλινδρόμησης μερικών ελαχίστων τετραγώνων |

# ABBREVIATIONS

**Table 2: List of abbreviations**

| | |
|---|---|
| WHO | World Health Organization |
| FDA | Food and Drug Administration |
| FAOSTAT | Food and Agriculture Organization Corporate Statistical Database |
| CDC | Centers of Disease Control and Prevention |
| AMD | Age-related Macular Degeneration |
| SFE | Supercritical fluid extraction |
| ASE | Accelerated solvent extraction |
| PLE | Pressurized liquid extraction |
| UAE | Ultrasound-assisted extraction |
| MAE | Microwave-assisted extraction |
| SWE | Subcritical water extraction |
| PEFE | Pulsed electric field extraction |
| SPME | Solid phase microextraction |
| SBSE | Stir bar sorptive extraction |
| LPME | Liquid phase microextraction |
| DLLME | Dispersive liquid–liquid microextraction |
| HFRLMs | Hollow fiber renewal liquid membranes |
| HFSD | Hollow fiber strip dispersion |
| COSMO-RS | Conductor-like screening model for real solvents |
| QSPR | Quantitative structure–properties relationship |
| NADES | Natural deep eutectic solvents |
| US | Ultrasounds |
| GRAS | Recognized-as-safe solvents |
| MW | Microwaves |
| SFME | Solvent-free microwave extraction |
| PSFME | Pressurized solvent-free microwave extraction |
| VMHD | Vacuum microwave hydro distillation |
| ISFME | Improved solvent-free microwave extraction |
| MSDf | Microwave steam diffusion |
| MHG | Microwave hydrodiffusion and gravity |

| | |
|---|---|
| MDG | Microwave dry-diffusion and gravity |
| ILs | Ionic liquids |
| DES | Deep eutectic solvents |
| UMAE | Ultrasound-microwave assisted extraction |
| SF | Supercritical fluids |
| UASFE | Ultrasound-assisted supercritical fluid extraction |
| SPE | Solid phase extraction |
| DOE | Design of experiments/Experimental design |
| LC-MS | Liquid chromatography-mass spectrometry |
| GC-MS | Gas chromatography-mass spectrometry |
| RSM | Response surface methodology |
| CCD | Central composite design |
| BBD | Box-Behnken design |
| PHW | Pressurized hot water |
| EOs | Essential oils |
| UPLC | Ultra performance liquid chromatography |
| NMR | Nuclear magnetic resonance |
| APCI | Atmospheric pressure chemical ionization |
| PDA | Photo diode array |
| PCA | Principal component analysis |
| OPLS-DA | Orthogonal partial least square-Discriminant analysis |
| MTBE | Methyl-*tert*-butyl ether |
| DMF | N,N-Dimethylformamide |
| FMAE | Focused microwave assisted extraction |
| ESI | Electrospray ionization |
| TGA | Thermogravimetric analysis |
| DSC | Differential scanning calorimetry |
| 1D-NOESY | One dimension-Nuclear Overhauser effect spectroscopy |
| gCOSY | Gradient correlation spectroscopy |
| gHSQCad | Gradient heteronuclear single quantum coherence with adiabatic pulses |
| gHMBCad | Gradient heteronuclear multiple bond coherence with adiabatic pulses |
| TSP | 3-(Trimethylsilyl)propionic-2,2,3,3-$d_4$ acid sodium salt |

| | |
|---|---|
| COW | Correlation optimized warping |
| ROC | Receiver operating characteristic |
| DMSO | Dimethyl sulfoxide |
| ANOVA | Analysis of variance |
| SS | Sum of squares |
| IS | Internal standard |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| ICH | International council for harmonisation of technical requirements for pharmaceuticals for human use |
| SRM | Selected reaction monitoring |
| RT | Retention time |
| ME | Matrix effect |
| QC | Quality control |
| CV | Coefficient of variation |
| RSD | Relative standard deviation |
| RE | Relative error |

# APPENDIX I. SUPPLEMENTARY DATA-FIGURES

**Figure S1. *vis*-Spectra of carotenoids standards. (a) b-Carotene (b) Lutein (c) Zeaxanthin (d) Astaxanthin (d) Canthaxanthin.**

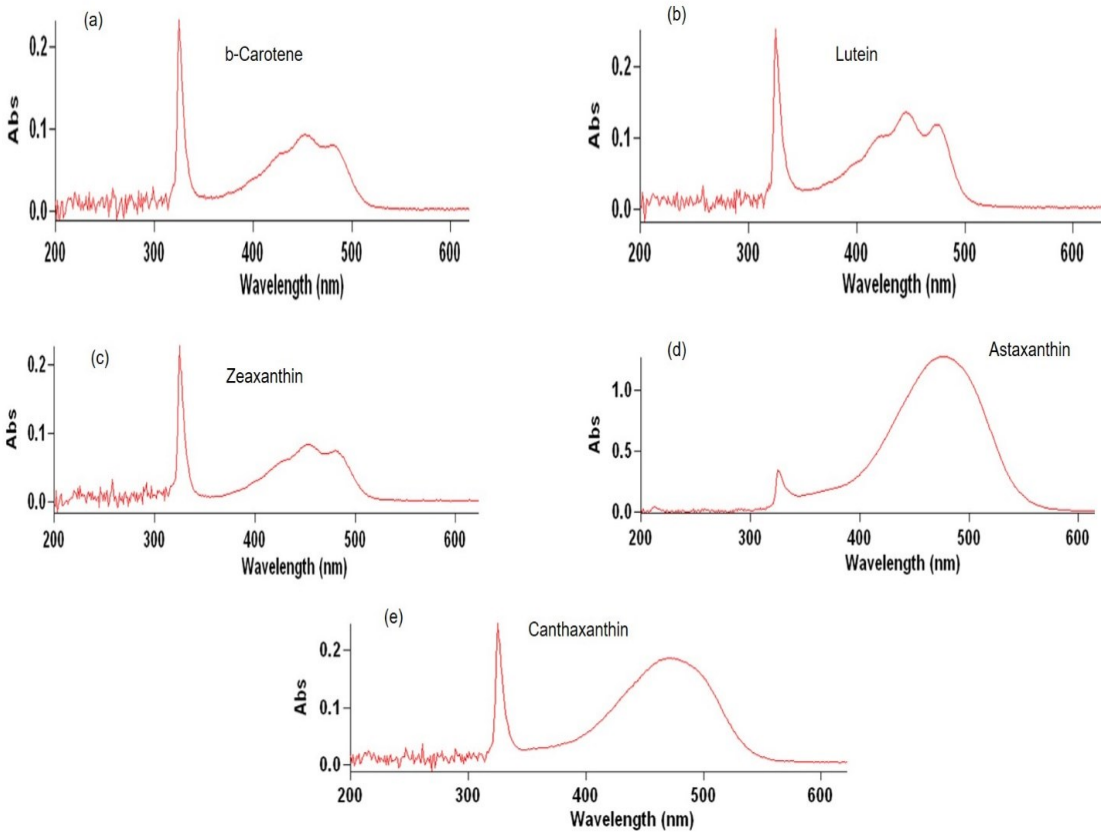


**Figure S2. Plackett-Burman design: Normal probability plot of the effect of APCI parameters on b-carotene intensity.**

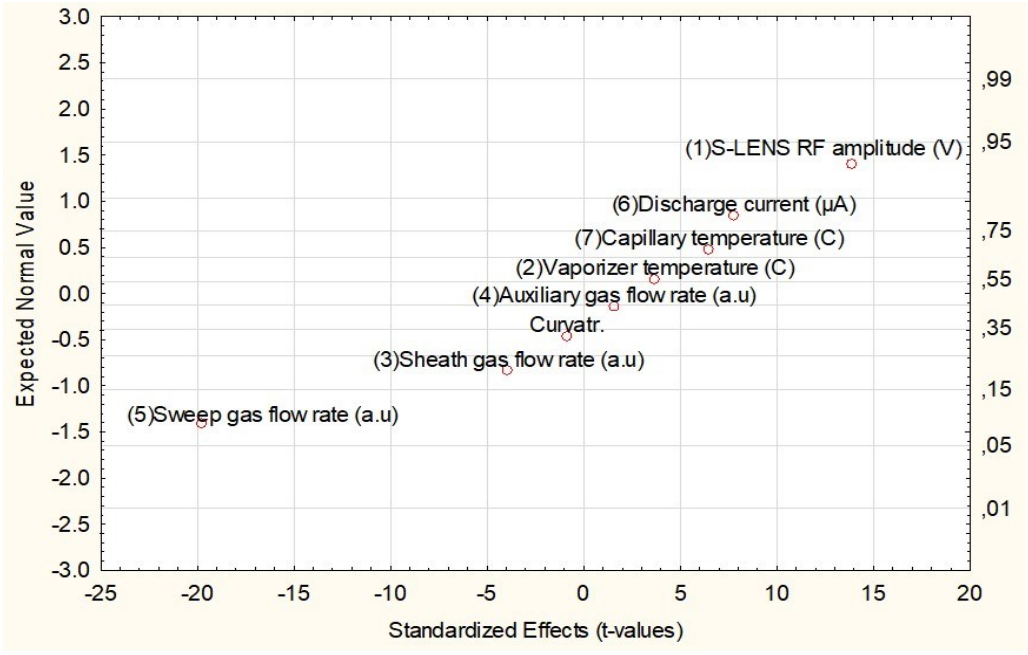


**Figure S3. Plots of observed versus predicted values of BBD for (a) UAE and (b) MAE of apricots pulp.**

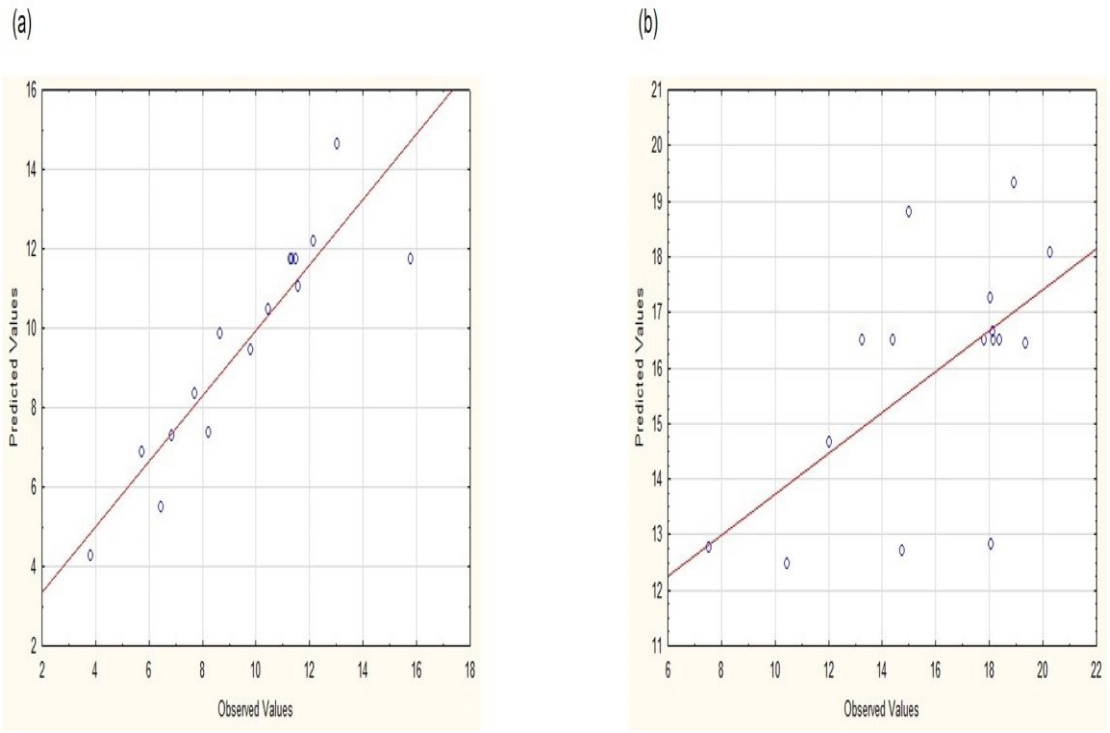


**Figure S4. Plots of observed versus predicted values of BBD for (a) UAE and (b) MAE of egg yolk.**

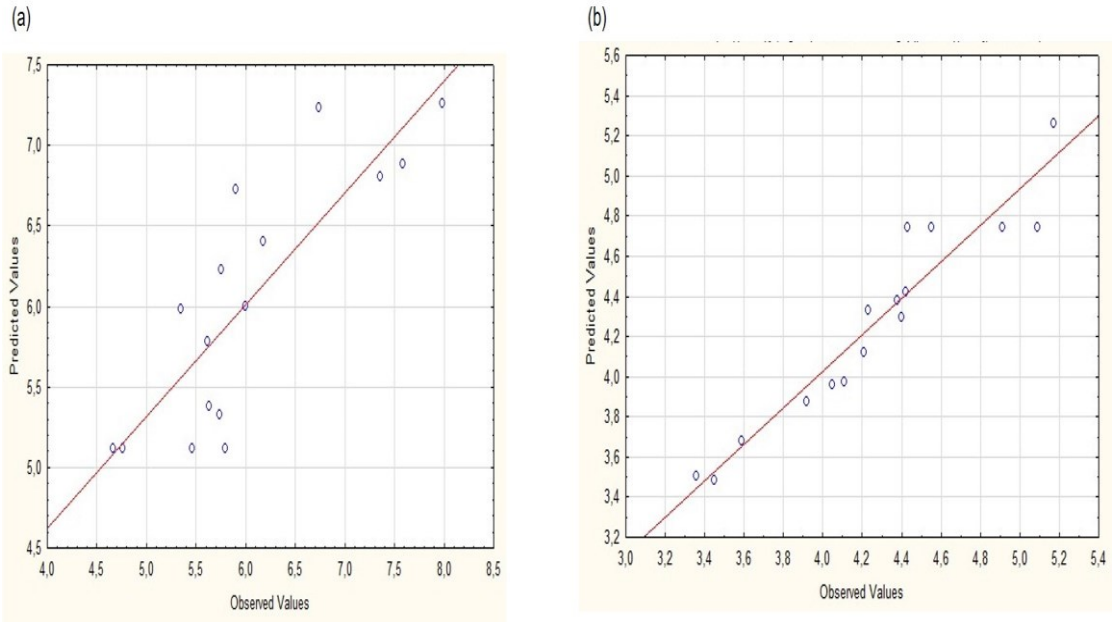


**Figure S5. Plots of observed versus predicted values of BBD for (a) UAE and (b) MAE of shrimp body.**

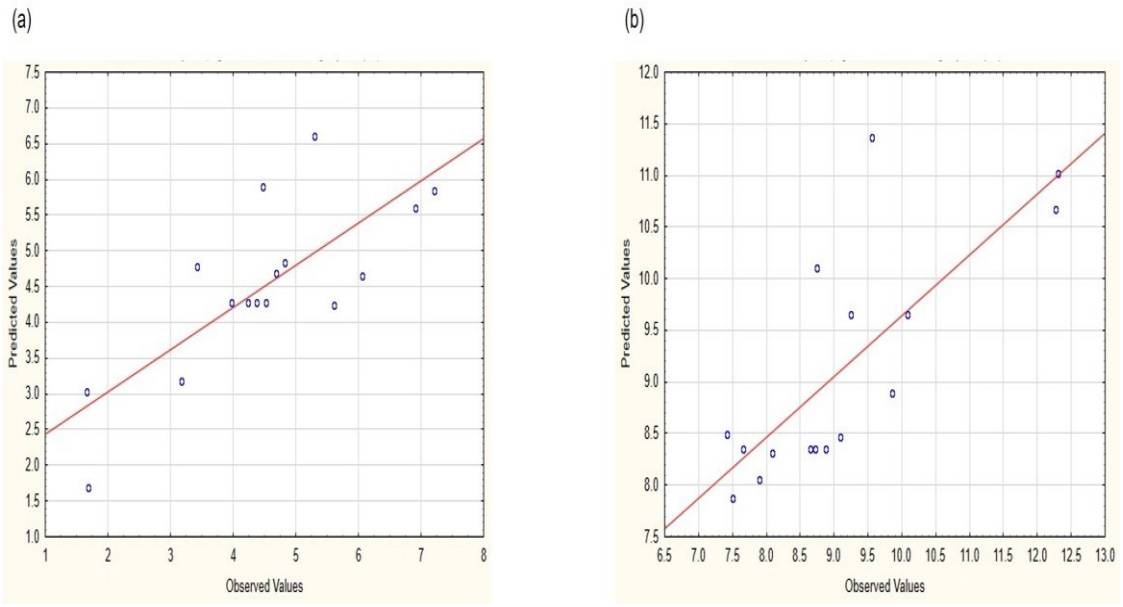


**Figure S6. NOESY spectra of CC/TA in DMSO-$d_6$ [177].**

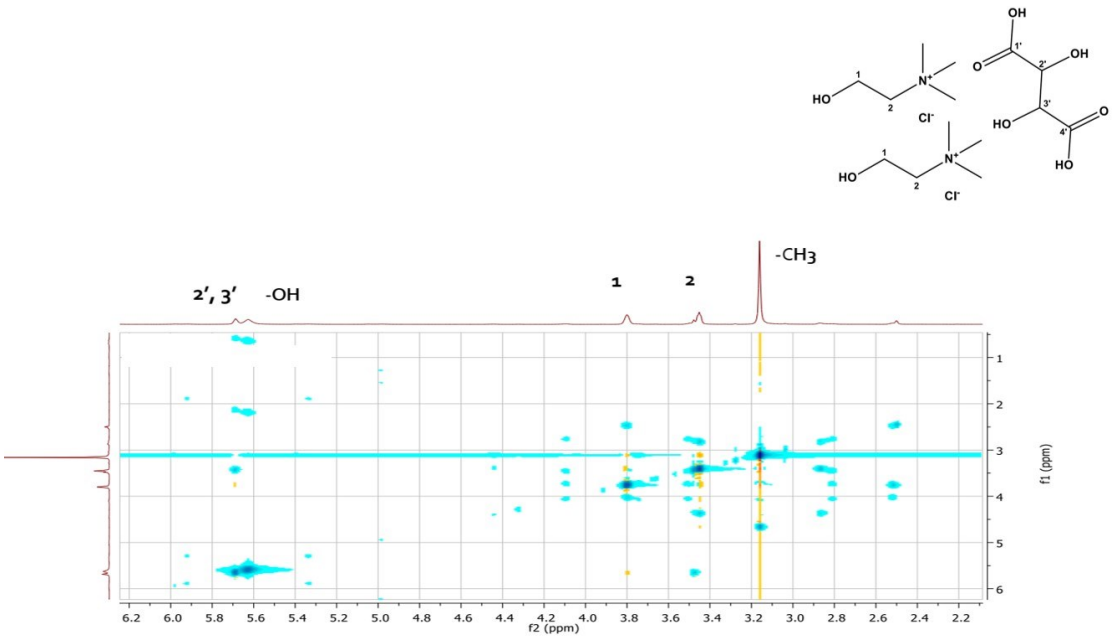


**Figure S7. TGA and DSC spectra of (a) physical mixture of the components and (b) CC/TA NADES [177].**

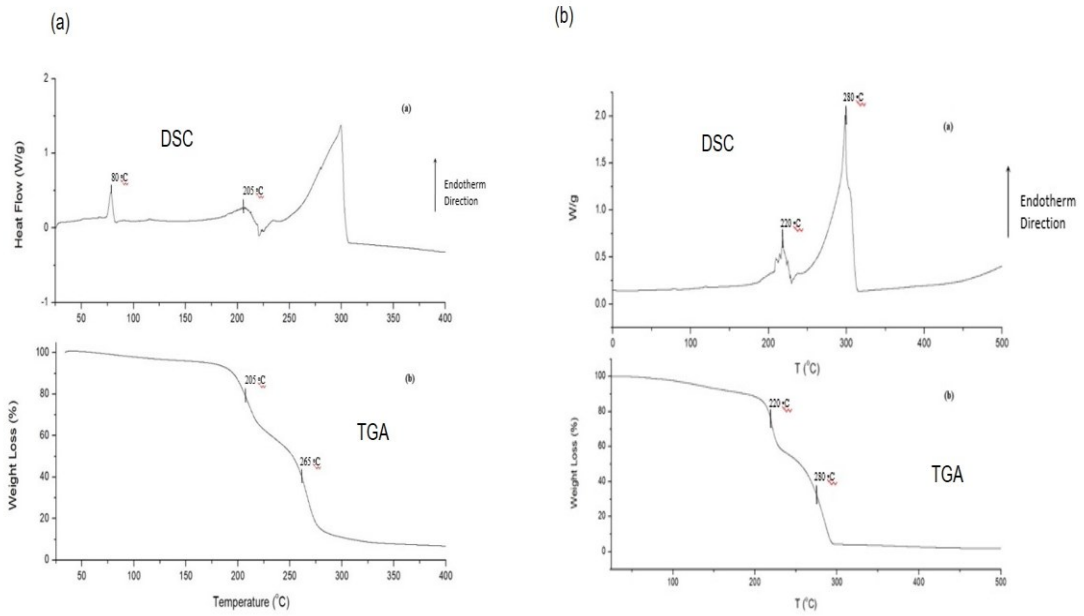


**Figure S8. Retention times and mass transitions of carotenoids of apricot byproducts' MAE optimal extract.**

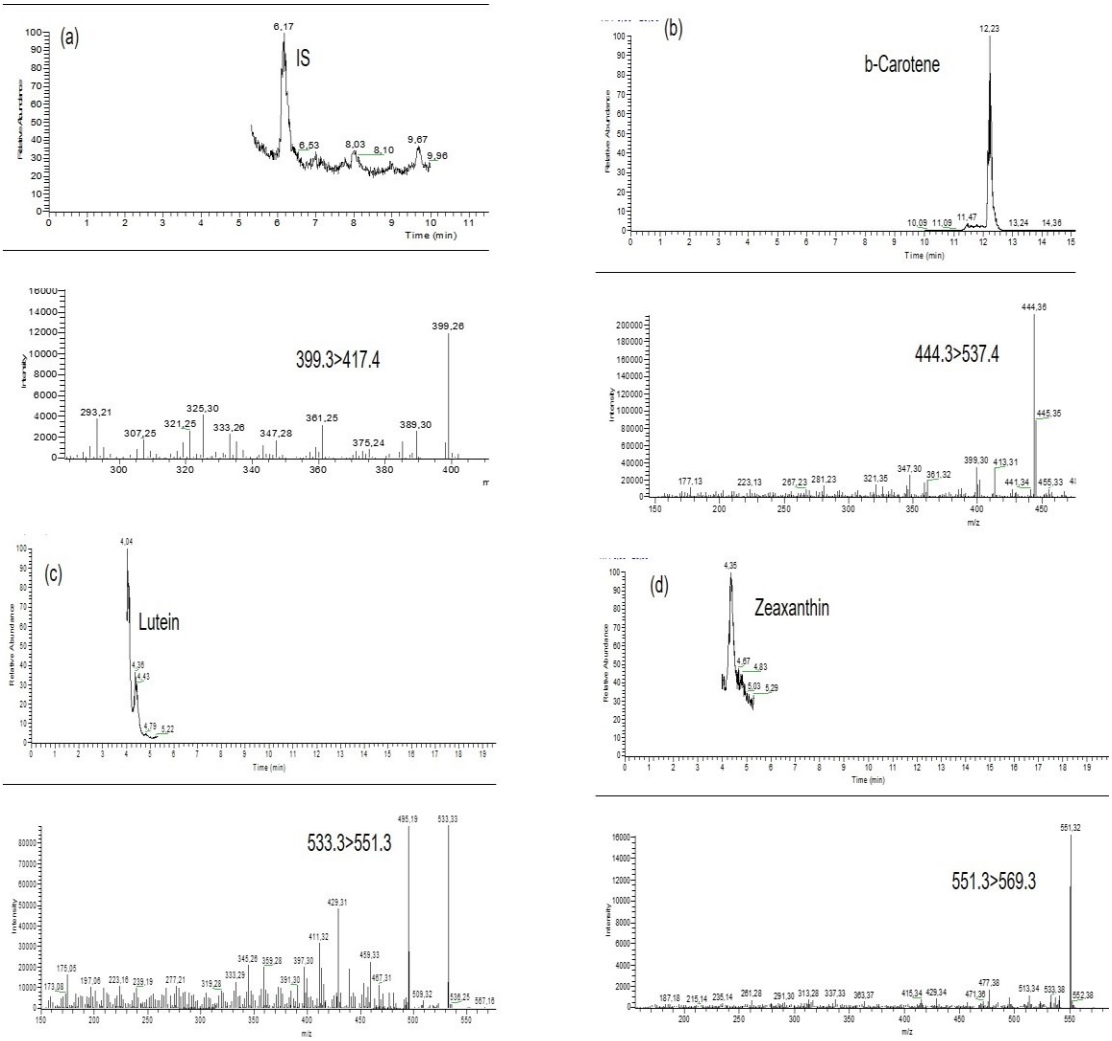


**Figure S9. Retention times and mass transitions of carotenoids of egg yolk's UAE optimal extract.**

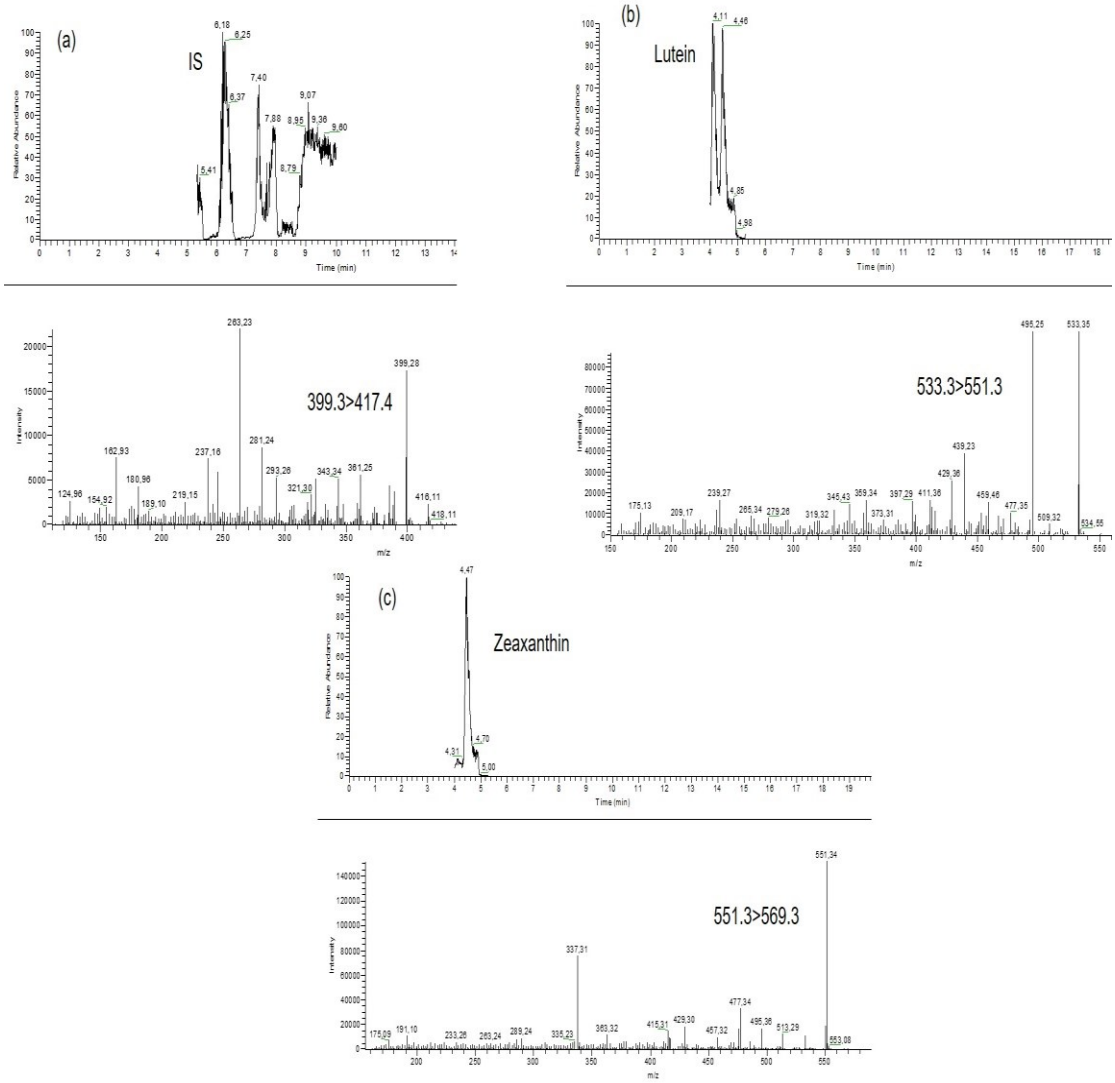


**Figure S10. Canthaxanthin MS/MS peak and product ion in optimized UAE egg yolk extract.**

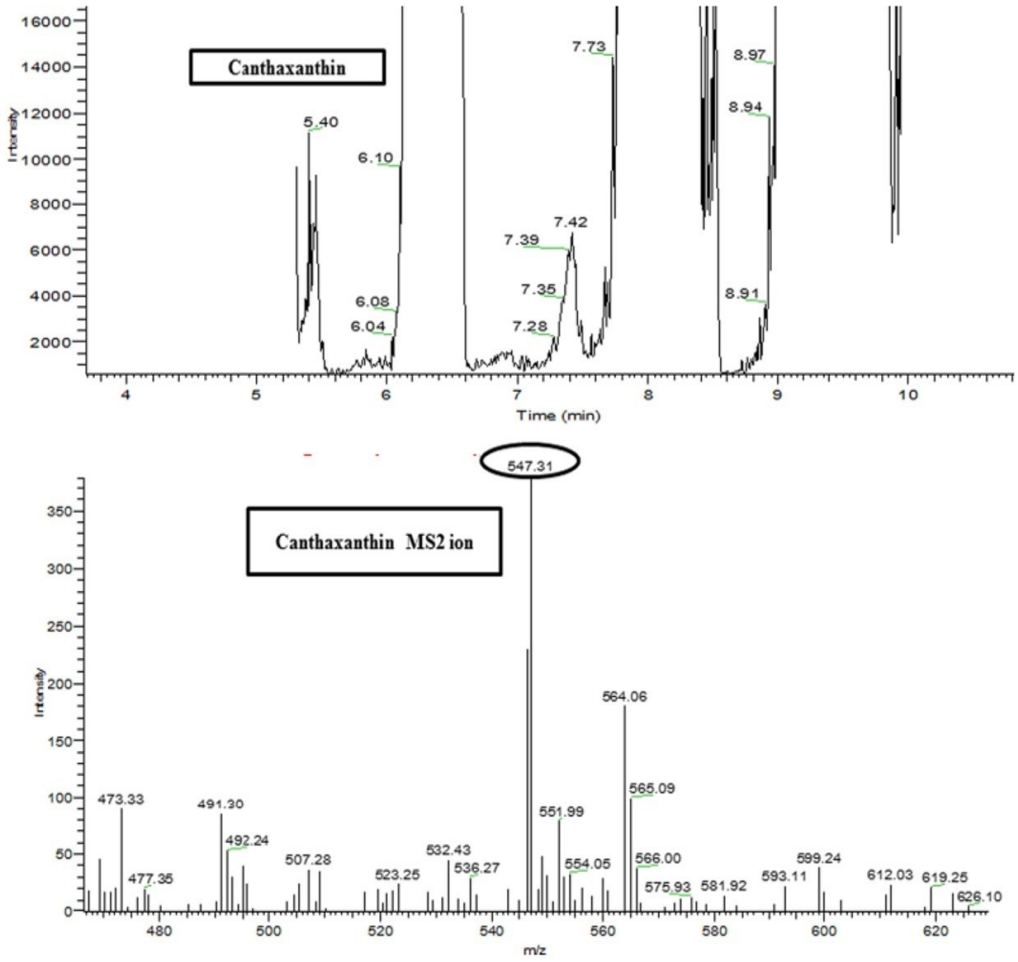


**Figure S11. NMR spectrum of UAE egg yolk extract ($^1$H NMR in $CDCl_3$, Varian 600 MHz).**

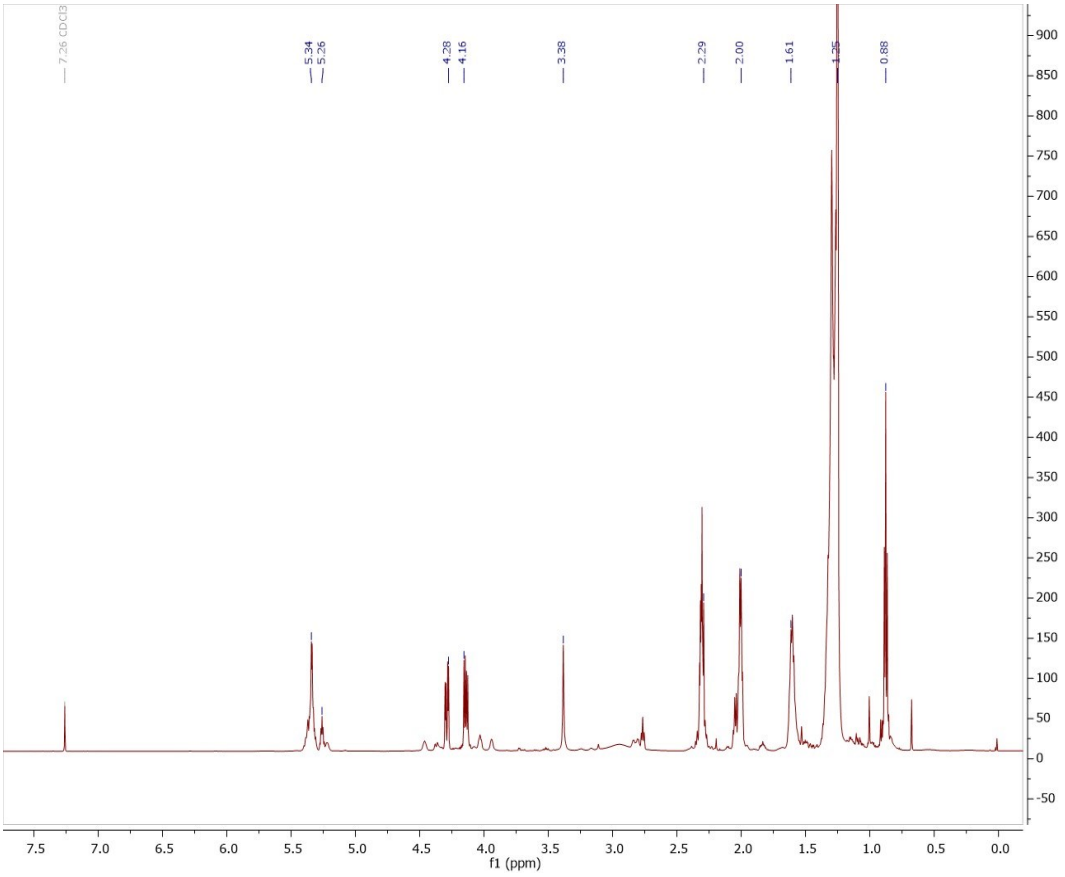


**Figure S12. Retention times and mass transitions of carotenoids of shrimp head's UAE optimal extract.**

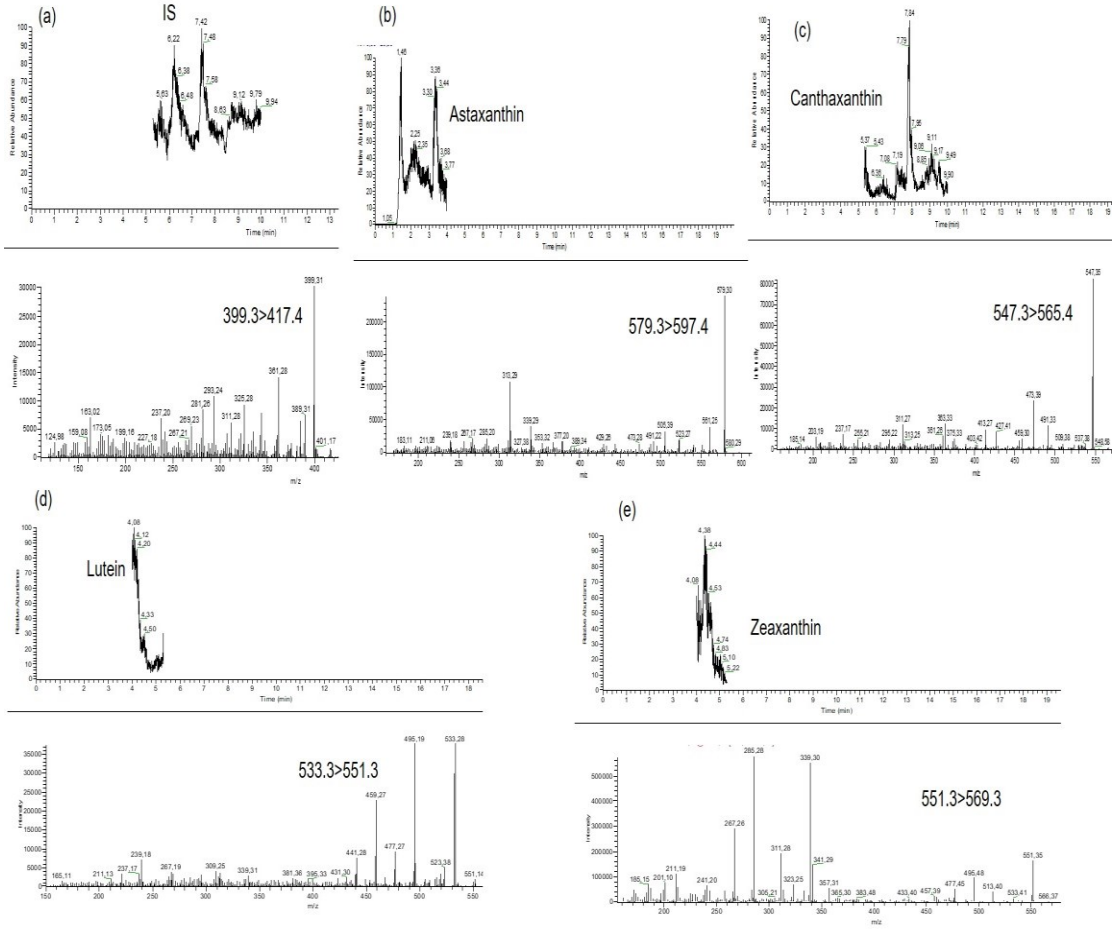


**Figure S13. Retention times and mass transitions of carotenoids of shrimp body's MAE optimal extract.**

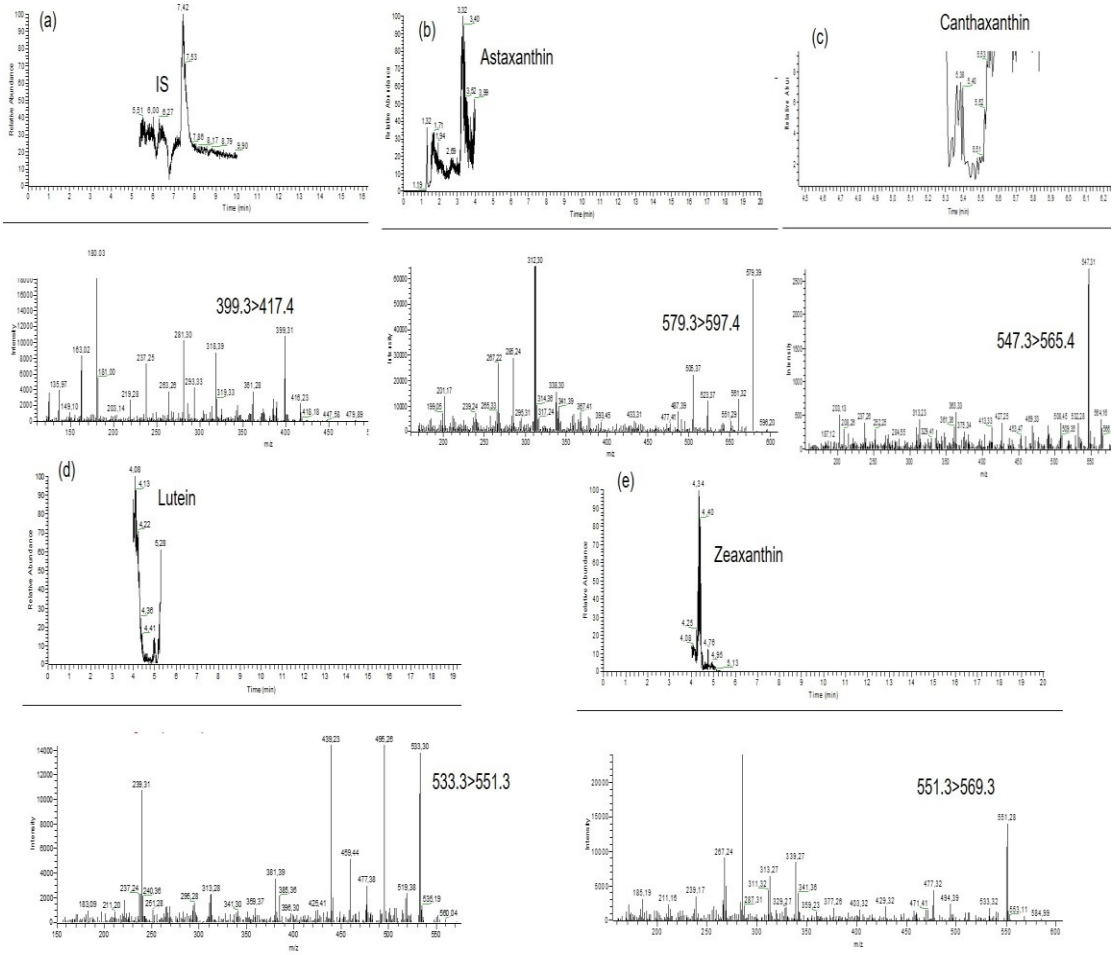
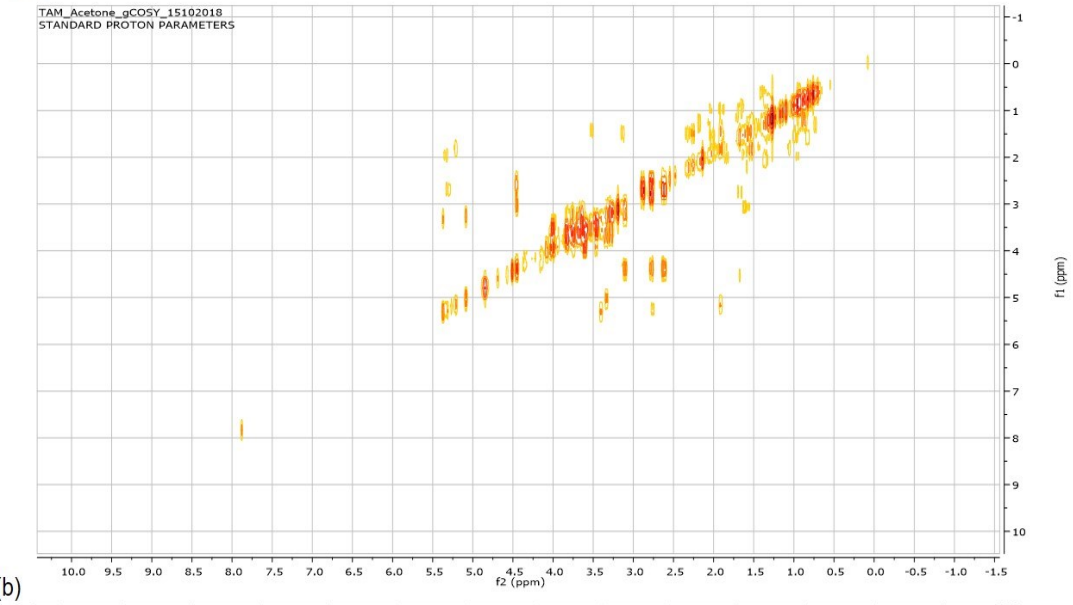


**Figure S14. 2D spectra of a characteristic extract of Group 1 (MAE_acetone) (a) gCOSY (b) gHSQCad.**

(a)




(b)

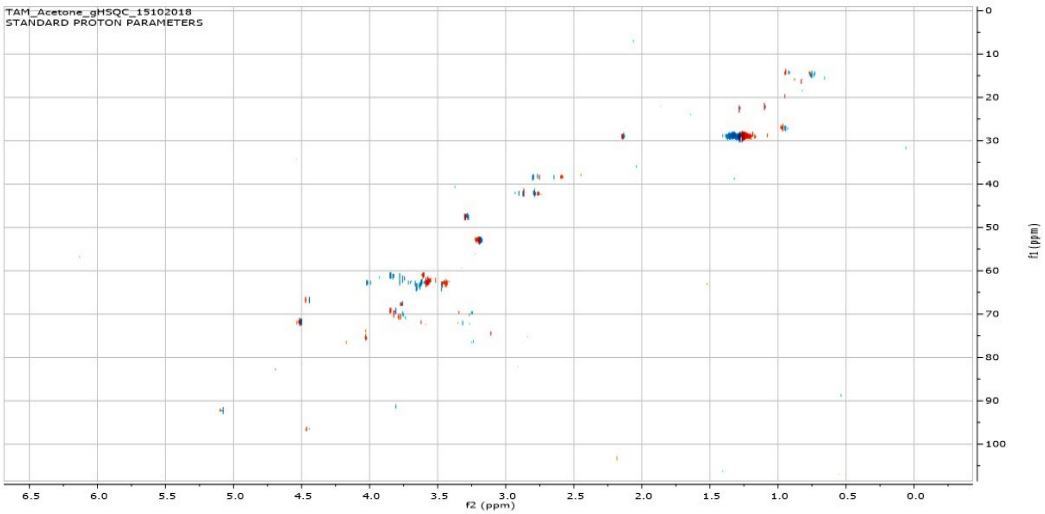
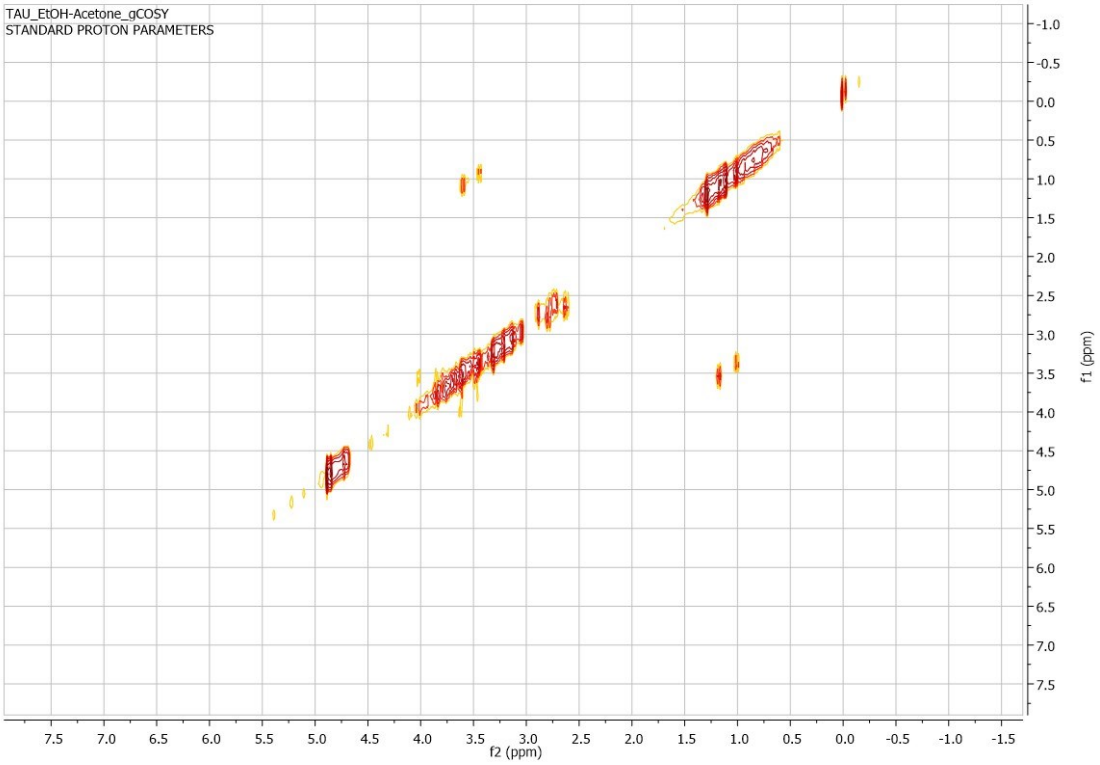


**Figure S15. 2D spectra of a characteristic extract of Group 2 (UAE_ethanol-acetone) (a) gCOSY (b) gHSQCad.**

(a)




(b)

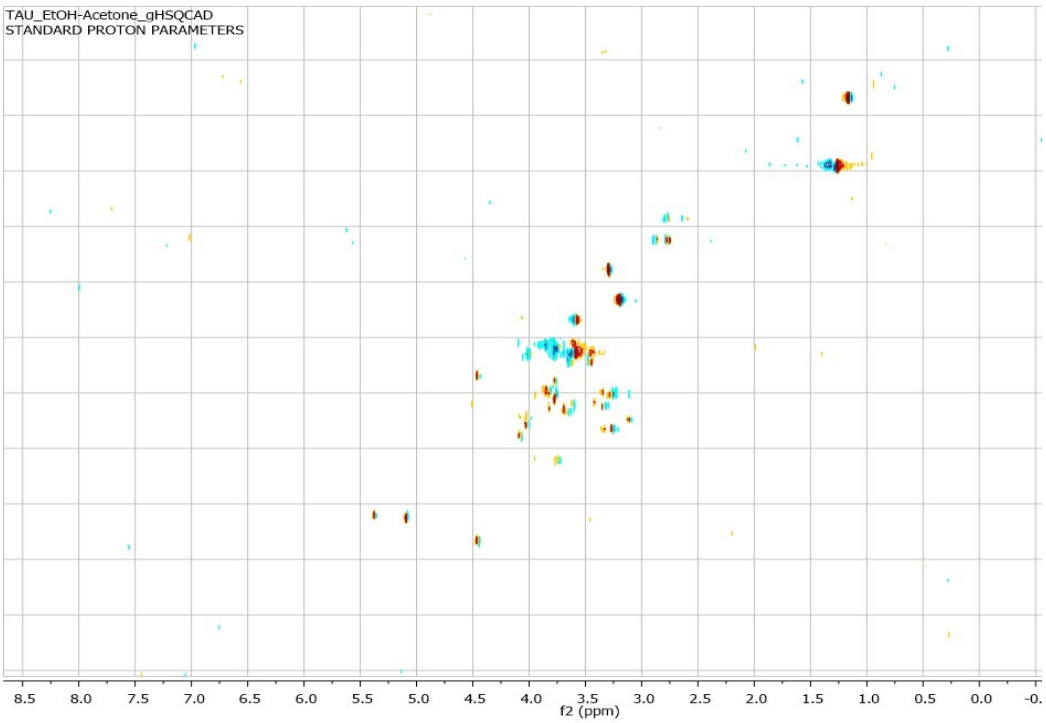
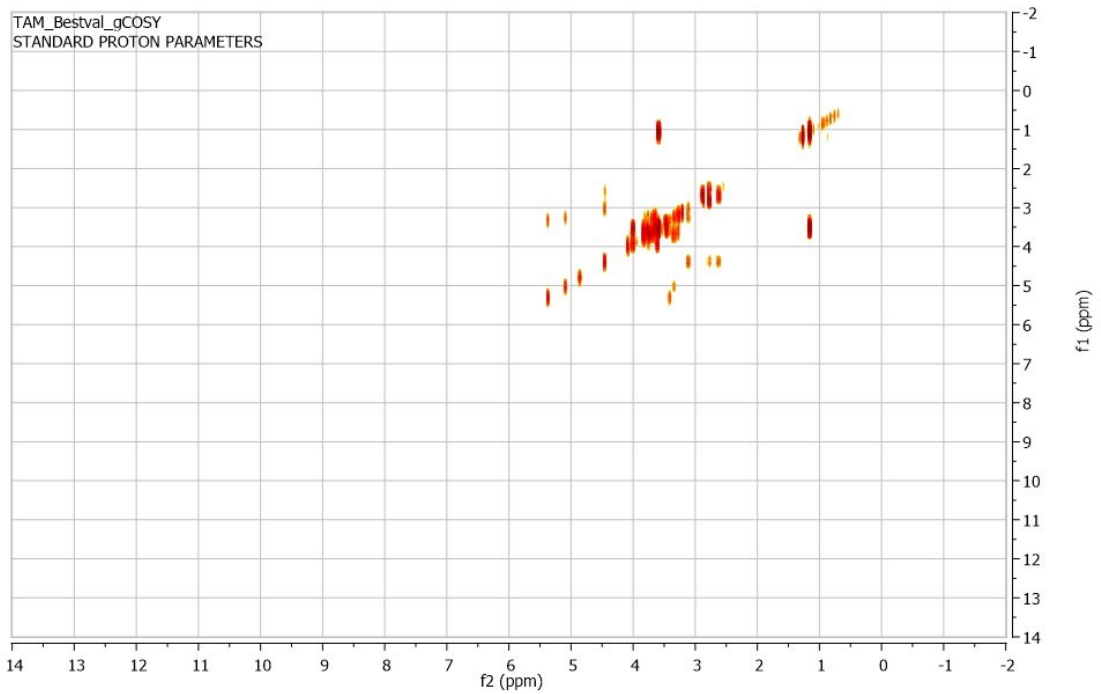


**Figure S16. 2D spectra of a characteristic extract of Group 3 (MAE_optimal extract) (a) gCOSY (b) gHSQCad.**

(a)




(b)

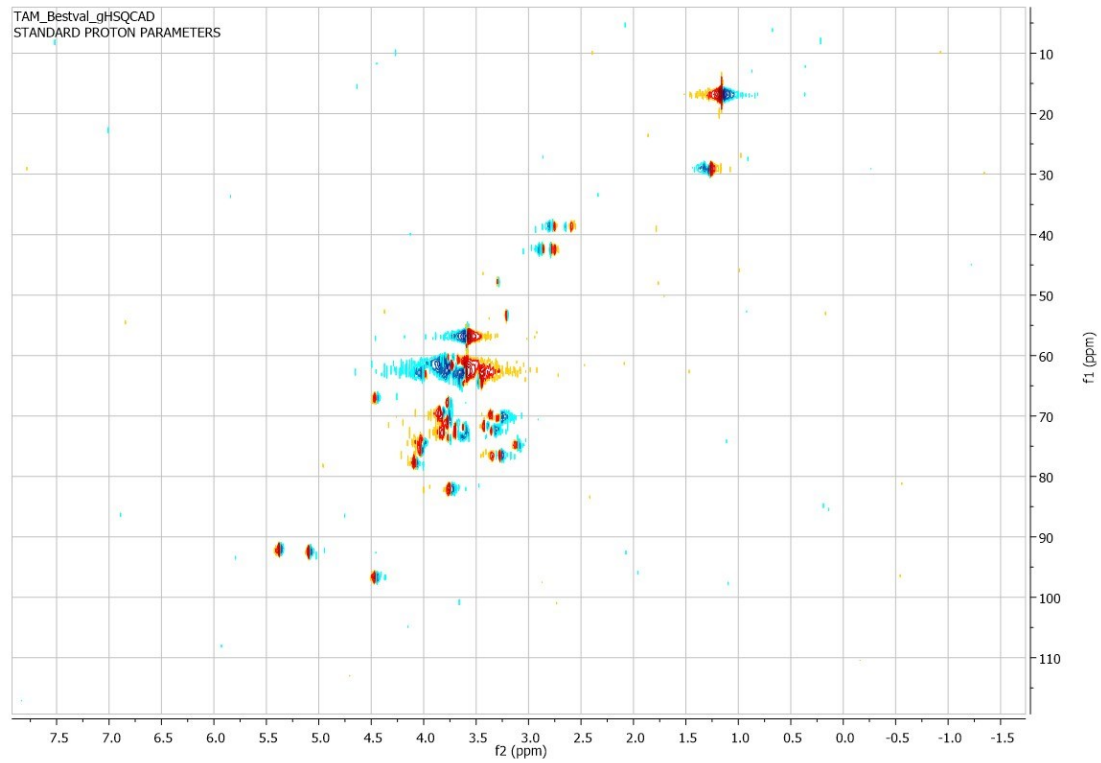
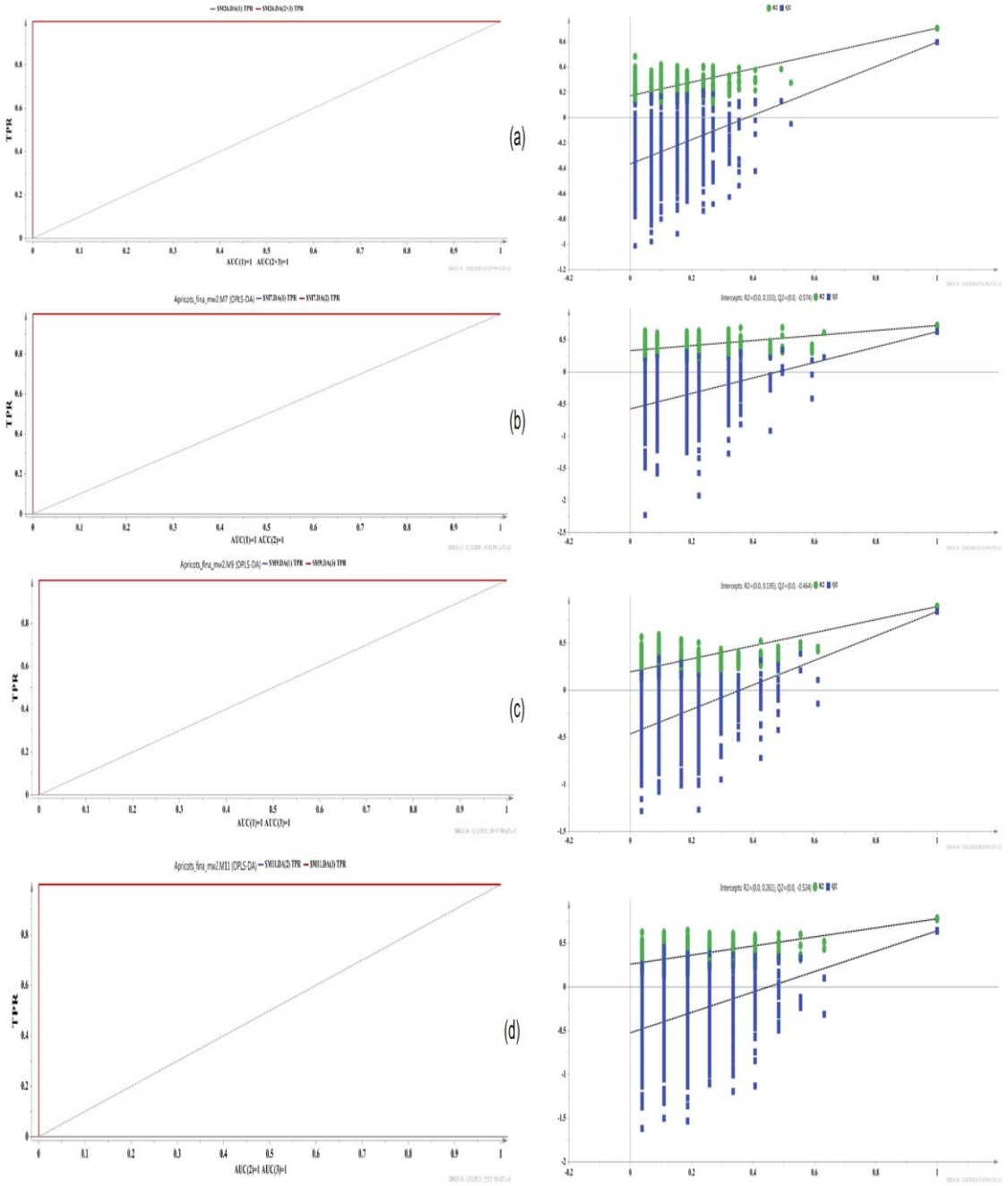


**Figure S17. Permutation testing and ROC curves of OPLS-DA models (a) Group 1 vs 2+3 (b) Group 1 vs 2 (c) Group 1 vs 3 (d) Group 2 vs 3.**



# APPENDIX II. SUPPLEMENTARY DATA-TABLES

**Table S1.Plackett-Burman design: Coded and real values of APCI parameters**

| **MS factors** | | ***Coded values*** | |
|---|---|---|---|
| | -1 | 0* | 1 |
| | | ***Real values*** | |
| **S-LENS RF Amplitude (V)** | 55 | 63 | 70 |
| **Vaporizer Temperature (°C)** | 300 | 375 | 450 |
| **Sheath Gas Flow rate (a.u.)** | 25 | 38 | 50 |
| **Auxiliary Gas Flow Rate (a.u.)** | 10 | 8 | 5 |
| **Sweep Gas Flow Rate (a.u.)** | 10 | 8 | 5 |
| **Discharge Current (µA)** | 9 | 7 | 5 |
| **Capillary Temperature (°C)** | 150 | 225 | 300 |

** Center points added to two-level Plackett-Burman design to detect curvature in the response and to evaluate variability by avoiding repetitions at the corner points*

**Table S2. UAE and MAE extraction yields of different solvent systems measured by APCI(+) LC-MS/MS. (a) Apricots pulp (b) Egg yolk (c) Shrimp head and body.**

**(a)**

| Extraction solvent (v/v) | Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev, n=3)* | |
|---|---|---|
| | **UAE** | **MAE** |
| **Chloroform-Methanol 1:1** | 9.39(±0.15) | 6.97(±0.27) |
| **Chloroform-Methanol 2:1** | 0.94(±0.092) | 3.33(±0.35) |
| **Chloroform** | 0.34(±0.10) | 1.192(±0.095) |
| **Ethanol-Acetone 1:1** | 6.13(±0.23) | 10.61(±0.86) |
| **Ethanol** | 5.03(±0.31) | 11.09(±0.98) |
| **Acetone** | 0.75(±0.17) | 0.48(±0.26) |
| ***n*-Hexane-Acetone 1:1** | 2.66(±0.20) | 0.84(±0.31) |
| ***n*-Hexane-Acetone-Ethanol 2:1:1** | 2.02(±0.067) | 0.27(±0.15) |

*n: number of sample replicates measured under repeatability conditions

**(b)**

| **Extraction solvent (v/v)** | **Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev, n=3)*** | |
|---|---|---|
| | **UAE** | **MAE** |
| **Chloroform** | 1.54(±0.14) | 4.09(±0.33) |
| ***n*-Hexane-Acetone 1:1** | 5.38(±0.21) | 3.48(±0.12) |
| **Chloroform-Methanol 2:1** | 4.10(±0.27) | 3.703(±0.08) |
| **Chloroform-Methanol 1:1** | 3.25(±0.17) | 2.61(±0.24) |
| **Ethanol** | 1.89(±0.26) | 3.26(±0.13) |

*n: number of sample replicates measured under repeatability conditions

**(c)**

| Extraction solvent (v/v) | Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev, n=3)* | |
|---|---|---|
| | **UAE** | **MAE** |
| **Chloroform** | 3.62 (±0.28) | 1.12 (±0.20) |
| **Chloroform-Methanol 1:1** | 2.96 (±0.19) | 2.52 (±0.15) |
| ***n*-Hexane-Acetone 1:1** | 5.18 (±0.21) | 1.87 (±0.24) |
| ***n*-Hexane-IPA 1:1** | 4.68 (±0.27) | 2.36 (±0.33) |
| **Acetone** | 5.82 (±0.31) | 4.14 (±0.37) |
| **Ethanol** | 4.74 (±0.36) | 6.05 (±0.25) |
| ***n*-Hexane:Acetone:Ethanol 2:1:1** | 5.38 (±0.13) | 8.66 (±0.18) |
| **Petroleum ether:Acetone:Ethanol 2:1:1** | 5.52 (±0.22) | 7.36 (±0.11) |

*n: number of sample replicates measured under repeatability conditions

**Table S3. Randomized experimental runs and carotenoid extraction yield of $2^3$ models for apricot pulp.**

| Standard run | Coded combinations($x_1$, $x_2$, $x_3$) | Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) | |
|---|---|---|---|
| ***$2^3$design*** | | | |
| | | **UAE** | **MAE** |
| **2** | -1,-1,+1 | 5.65 | 23.80 |
| **3** | -1,+1,-1 | 8.53 | 16.05 |
| **6** | +1,-1,+1 | 7.84 | 19.19 |
| **4** | -1,+1,+1 | 15.91 | 14.00 |
| **5** | +1,-1,-1 | 4.50 | 13.01 |
| **8** | +1,+1,+1 | 29.18 | 11.76 |
| **1** | -1,-1,-1 | 6.64 | 12.14 |
| **7** | +1,+1,-1 | 3.21 | 16.29 |
| ***BBD model*** | | | |
| | | **UAE** | **MAE** |
| **16** | 0,0,0 | 11.35 | 18.37 |
| **4** | -1,+1,0 | 5.74 | 18.91 |
| **12** | 0,+1,+1 | 8.65 | 15.00 |
| **3** | -1,+1,0 | 10.45 | 17.81 |
| **13** | 0,0,0 | 15.77 | 13.26 |
| **6** | +1,0,-1 | 8.22 | 20.27 |
| **11** | 0,-1,+1 | 3.82 | 18.06 |
| **8** | +1,0,+1 | 6.46 | 18.05 |
| **14** | 0,0,0 | 11.29 | 14.40 |
| **1** | -1,-1,0 | 11.56 | 14.74 |
| **10** | 0,+1,-1 | 9.79 | 19.35 |
| **15** | 0,0,0 | 11.48 | 18.16 |
| **7** | -1,0,+1 | 12.15 | 18.11 |
| **9** | 0,-1,-1 | 13.05 | 7.52 |
| **5** | -1,0,-1 | 6.86 | 12.03 |
| **2** | +1,-1,0 | 7.70 | 10. |

**Table S4. ANOVA table of (a) $2^3$ design and (b) BBD model for UAE and MAE of apricot pulp carotenoids.**

**(a)**

| Factors | | Sum of squares (SS) | | *F*-value | | *p*-value | |
|---|---|---|---|---|---|---|---|
| **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** |
| $x_2$ | $x_1$ | *129.69* | 4.13 | *9.45* | 5.45 | *0.054* | 0.15 |
| $x_3$ | $x_2$ | *159.38* | 12.57 | *11.61* | 16.59 | *0.042*[a] | 0.055 |
| $x_1x_3$ | $x_3$ | *65.70* | 15.83 | *4.79* | 20.89 | *0.12* | 0.045[a] |
| $x_2x_3$ | $x_1x_3$ | *120.13* | 7.93 | *8.75* | 10.47 | *0.060* | 0.084 |
| | $x_2x_3$ | | 74.54 | | 98.36 | | 0.010[a] |
| | | **UAE** | **MAE** | | | | |
| **Pure error (degrees of freedom)** | | *41.17 (3)* | 1.52 (3) | | | | |
| **Total SS (degrees of freedom)** | | *516.09 (7)* | 116.52 (7) | | | | |

[a]:Factors with *p*-value≤0.05

**(b)**

| Factors | | Sum of squares (SS) | | *F*-value | | *p*-value | |
|---|---|---|---|---|---|---|---|
| **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** |
| $x_1^2$ | $x_1^2$ | *16.10* | 1.63 | *5.93* | 0.54 | *0.051* | 0.48 |
| $x_1$ | $x_2$ | *23.06* | 58.20 | *8.49* | 19.18 | *0.027*[a] | 0.0024[a] |
| $x_2^2$ | $x_2^2$ | *10.24* | 18.83 | *3.77* | 6.20 | *0.10* | 0.037[a] |
| $x_3^2$ | $x_1x_2$ | *16.69* | 7.97 | *6.14* | 2.63 | *0.048*[a] | 0.14 |
| $x_1x_2^2$ | $x_1x_2^2$ | *2.23* | 22.20 | *0.82* | 7.32 | *0.43* | 0.027[a] |
| $x_1^2x_2$ | $x_1x_3$ | *1.94* | 24.16 | *0.71* | 7.96 | *0.40* | 0.022[a] |
| $x_1x_3$ | $x_2x_3$ | *12.41* | 58.43 | *4.57* | 19.25 | *0.076* | 0.0023[a] |
| $x_1^2x_3$ | | *29.41* | | *10.83* | | *0.017*[a] | |
| $x_2x_3$ | | *16.37* | | *6.03* | | *0.04* [a] | |
| | | **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** |
| **Lack of fit (degrees of freedom)** | | *1.77* *(3)* | 3.95 (5) | 0.12 (3) | 0.12 (5) | *0.941* *(3)* | 0.979 (5) |
| **Pure error (degrees of freedom)** | | *14.52* *(3)* | 20.32 (3) | | | | |
| **Total SS (degrees of freedom)** | | *142.52* *(15)* | 195.45 (15) | | | | |

[a]:Factors with *p*-value≤0.05

**Table S5. Predicted and observed extraction yields of apricot pulp at potential optimal experimental combinations.**

| ***UAE*** | **Extraction time (min)** | **US power (W)** | **Solvent/material ratio (mL $g^{-1}$)** | **Predicted extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample)** | **Experimental extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev)** |
|---|---|---|---|---|---|
| **Run A** | 15 | 600 | 25 | 12.43 | 8.46(±0.21) |
| **Run B** | 10 | 600 | 35 | 10.83 | 11.12(±0.34)[a] |
| **Run C** | 20 | 600 | 25 | 13.75 | 9.38(±0.15) |
| ***MAE*** | **Extraction time (min)** | **MW power (W)** | **Solvent/material ratio (mL $g^{-1}$)** | **Predicted extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample)** | **Experimental extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev)** |
| **Run A** | 20 | 120 | 45 | 19.40 | 19.28(±0.27)[a] |
| **Run B** | 20 | 120 | 60 | 19.51 | 17.22(±0.38) |
| **Run C** | 20 | 140 | 60 | 18.98 | 15.29(±0.26) |

*[a]: Optimal UAE and MAE experimental combinations

**Table S6. Randomized experimental runs and carotenoid extraction yield of $2^3$ models for egg yolk.**

| Standard run | Coded combinations($x_1$, $x_2$, $x_3$) | Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) | |
|---|---|---|---|
| ***$2^3$design*** | | | |
| | | **UAE** | **MAE** |
| **2** | -1,-1,+1 | 6.47 | 4.34 |
| **3** | -1,+1,-1 | 2.99 | 4.54 |
| **6** | +1,-1,+1 | 6.61 | 3.54 |
| **4** | -1,+1,+1 | 6.67 | 4.40 |
| **5** | +1,-1,-1 | 3.57 | 3.91 |
| **8** | +1,+1,+1 | 7.02 | 3.98 |
| **1** | -1,-1,-1 | 4.97 | 4.33 |
| **7** | +1,+1,-1 | 6.04 | 4.40 |
| ***BBD model*** | | | |
| | | **UAE** | **MAE** |
| **16** | 0,0,0 | 5.79 | 5.09 |
| **4** | -1,+1,0 | 7.58 | 4.11 |
| **12** | 0,+1,+1 | 6.18 | 4.42 |
| **3** | -1,+1,0 | 5.75 | 3.45 |
| **13** | 0,0,0 | 5.46 | 4.43 |
| **6** | +1,0,-1 | 5.35 | 3.59 |
| **11** | 0,-1,+1 | 5.99 | 4.38 |
| **8** | +1,0,+1 | 6.74 | 5.17 |
| **14** | 0,0,0 | 4.66 | 4.91 |
| **1** | -1,-1,0 | 5.90 | 3.36 |
| **10** | 0,+1,-1 | 5.62 | 4.23 |
| **15** | 0,0,0 | 4.76 | 4.55 |
| **7** | -1,0,+1 | 7.98 | 4.21 |
| **9** | 0,-1,-1 | 5.63 | 4.40 |
| **5** | -1,0,-1 | 7.36 | 4.05 |
| **2** | +1,-1,0 | 5.74 | 3.92 |

**Table S7. ANOVA table of (a) $2^3$ design and (b) BBD model for UAE and MAE of egg yolk carotenoids.**

**(a)**

| Factors | | Sum of squares (SS) | | *F*-value | | *p*-value | |
|---|---|---|---|---|---|---|---|
| **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** |
| $x_1$ | $x_1$ | *0.66* | 0.40 | *0.92* | 316.84 | *0.41* | 0.036[a] |
| $x_2$ | $x_2$ | *0.20* | 0.18 | *0.28* | 144.00 | *0.63* | 0.0053 |
| $x_3$ | $x_3$ | *11.02* | 0.10 | *15.22* | 84.64 | *0.030*[a] | 0.069 |
| $x_1x_2$ | $x_1x_2$ | *2.92* | 0.054 | *4.03* | 43.56 | *0.14* | 0.096 |
| | $x_1x_3$ | | 0.054 | | 43.56 | | 0.096 |
| | $x_2x_3$ | | 0.0050 | | 4.00 | | 0.30 |
| | | **UAE** | **MAE** | | | | |
| **Pure error (degrees of freedom)** | | *2.17 (3)* | 0.0012(3) | | | | |
| **Total SS (degrees of freedom)** | | *16.98 (7)* | 0.78 (7) | | | | |

[a] Significant terms (*p*-value≤0.05)

**(b)**

| Factors | | Sum of squares (SS) | | *F*-value | | *p*-value | |
|---|---|---|---|---|---|---|---|
| **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** |
| $x_1^2$ | $x_1$ | *4.31* | 0.43 | *14.42* | 4.57 | *0.032*[a] | 0.12 |
| $x_2$ | $x_1^2$ | *0.63* | 1.30 | *2.12* | 13.69 | *0.24* | 0.034[a] |
| $x_3$ | $x_2$ | *1.23* | 0.0028 | *4.12* | 0.030 | *0.13* | 0.87 |
| $x_3^2$ | $x_2^2$ | *1.70* | 0.87 | *5.68* | 9.20 | *0.097*[b] | 0.056[b] |
| $x_1x_2$ | $x_3$ | *0.99* | 0.67 | *3.31* | 7.05 | *0.17* | 0.077[b] |
| $x_1x_2^2$ | $x_3^2$ | *3.34* | 0.025 | *11.17* | 0.26 | *0.044*[a] | 0.64 |
| $x_1^2x_2$ | $x_1x_2^2$ | *0.29* | 0.065 | *0.98* | 0.68 | *0.40* | 0.47 |
| $x_1x_3$ | $x_1x_3$ | *0.15* | 0.50 | *0.50* | 5.33 | *0.53* | 0.10 |
| $x_1^2x_3$ | $x_1^2x_3$ | *0.15* | 0.31 | *0.50* | 3.26 | *0.53* | 0.17 |
| | | **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** |
| **Lack of fit** | | *0.016* *(3)* | 0.034 (3) | *0.017* *(3)* | 0.12 (3) | *1.00* *(3)* | 0.94 (3) |
| **Pure error (degrees of freedom)** | | *0.90* *(3)* | 0.28 (3) | | | | |
| **Total SS (degrees of freedom)** | | *13.35* *(15)* | 4.21 (15) | | | | |

[a] Significant terms (*p*-value≤0.05), [b]Terms of medium significance (*p*-value≤0.1)

**Table S8. Predicted and observed extraction yields of egg yolk at potential optimal experimental combinations.**

| ***UAE*** | **Extraction time (min)** | **US power (W)** | **Solvent/material ratio (mL $g^{-1}$)** | **Predicted extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample)** | **Experimental extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev)** |
|---|---|---|---|---|---|
| **Run A** | 19 | 550 | 25 | 7.71 | 5.03(±0.28) |
| **Run B** | 22 | 600 | 30 | 5.62 | 4.71(±0.23) |
| **Run C** | 19 | 600 | 35 | 6.37 | 7.41(±0.34)[a] |
| ***MAE*** | **Extraction time (min)** | **MW power (W)** | **Solvent/material ratio (mL $g^{-1}$)** | **Predicted extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample)** | **Experimental extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev)** |
| **Run A** | 22 | 182 | 40 | 4.97 | 4.88(±0.18)[a] |
| **Run B** | 18 | 170 | 30 | 4.56 | 4.55(±0.12) |
| **Run C** | 28 | 190 | 30 | 4.46 | 4.02(±0.24) |

*[a]: Optimal UAE and MAE experimental combination

**Table S9. Randomized experimental runs and carotenoid extraction yield of $2^3$ models for shrimp body.**

| Standard run | Coded combinations($x_1$, $x_2$, $x_3$) | Extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) | |
|---|---|---|---|
| ***$2^3$design*** | | | |
| | | **UAE** | **MAE** |
| **2** | -1,-1,+1 | 4.02 | 7.22 |
| **3** | -1,+1,-1 | 8.48 | 9.62 |
| **6** | +1,-1,+1 | 5.23 | 7.78 |
| **4** | -1,+1,+1 | 6.75 | 7.46 |
| **5** | +1,-1,-1 | 6.62 | 7.85 |
| **8** | +1,+1,+1 | 7.70 | 8.29 |
| **1** | -1,-1,-1 | 6.64 | 11.66 |
| **7** | +1,+1,-1 | 7.04 | 8.36 |
| ***BBD model*** | | | |
| | | **UAE** | **MAE** |
| **16** | 0,0,0 | 3.98 | 8.64 |
| **4** | -1,+1,0 | 6.91 | 7.51 |
| **12** | 0,+1,+1 | 3.17 | 9.09 |
| **3** | -1,+1,0 | 5.30 | 7.90 |
| **13** | 0,0,0 | 4.37 | 8.88 |
| **6** | +1,0,-1 | 3.42 | 9.56 |
| **11** | 0,-1,+1 | 1.69 | 7.66 |
| **8** | +1,0,+1 | 1.66 | 8.75 |
| **14** | 0,0,0 | 4.24 | 7.42 |
| **1** | -1,-1,0 | 4.47 | 9.25 |
| **10** | 0,+1,-1 | 4.82 | 12.30 |
| **15** | 0,0,0 | 4.52 | 8.08 |
| **7** | -1,0,+1 | 5.61 | 8.72 |
| **9** | 0,-1,-1 | 4.69 | 9.86 |
| **5** | -1,0,-1 | 7.21 | 10.09 |
| **2** | +1,-1,0 | 6.06 | 12.28 |

**Table S10. ANOVA table of (a) $2^3$ design and (b) BBD model for UAE and MAE of shrimp body carotenoids.**

**(a)**

| **Factors** | | **Sum of squares (SS)** | | ***F*-value** | | ***p*-value** | |
|---|---|---|---|---|---|---|---|
| **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** |
| $x_1$ | $x_1$ | *3.23* | 5.68 | *28.23* | 12.41 | *0.034*[a] | 0.039[a] |
| $x_2$ | $x_3$ | *6.93* | 1.70 | *60.59* | 3.72 | *0.016*[a] | 0.15 |
| $x_1x_3$ | $x_1x_3$ | *1.09* | 5.20 | *9.55* | 11.35 | *0.091* | 0.043[a] |
| $x_1x_3$ | $x_2x_3$ | *1.64* | 0.94 | *14.30* | 2.17 | *0.063* | 0.24 |
| $x_2x_3$ | | *0.35* | | *3.07* | | *0.22* | |
| | | **UAE** | **MAE** | | | | |
| **Pure error (degrees of freedom)** | | *0.23 (3)* | 1.37 (3) | | | | |
| **Total SS (degrees of freedom)** | | *13.46 (7)* | 14.95 (7) | | | | |

[a] Significant terms (*p*-value≤0.05)

**(b)**

| Factors | | Sum of squares (SS) | | *F*-value | | *p*-value | |
|---|---|---|---|---|---|---|---|
| **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** |
| $x_1^2$ | $x_1^2$ | *0.090* | 0.54 | *1.71* | 1.76 | *0.28* | 0.28 |
| $x_1$ | $x_2^2$ | *5.24* | 0.21 | *100.03* | 0.70 | *0.021*[a] | 0.46 |
| $x_2^2$ | $x_2$ | *1.35* | 8.49 | *25.81* | 27.68 | *0.015*[a] | 0.013[a] |
| $x_2$ | $x_3^2$ | *0.27* | 0.55 | *5.26* | 1.78 | *0.10* | 0.27 |
| $x_3^2$ | $x_3$ | *6.46* | 1.10 | *123.30* | 3.61 | *0.016*[a] | 0.15 |
| $x_3$ | $x_1x_2$ | *3.59* | 1.59 | *68.50* | 5.19 | *0.037*[a] | 0.11 |
| $x_1x_2^2$ | $x_1x_2^2$ | *14.96* | 2.06 | *285.37* | 6.72 | *0.00045*[a] | 0.080 |
| $x_1x_3$ | $x_1^2x_2$ | *0.0064* | 9.45 | *0.12* | 30.82 | *0.75* | 0.011[a] |
| $x_1^2x_3$ | $x_1x_3$ | *0.21* | 0.42 | *3.97* | 1.36 | *0.14* | 0.33 |
| $x_2x_3$ | $x_1^2x_3$ | *0.45* | 2.62 | *8.69* | 8.54 | *0.060* | 0.061 |
| | $x_2x_3$ | | 4.90 | | 15.98 | | 0.028[a] |
| | | | | | | | |
| | | **UAE** | **MAE** | **UAE** | **MAE** | **UAE** | **MAE** |
| **Lack of fit** | | *0.00071* | 0.12 | *0.0068* | 0.39 | *0.99* | 0.58 |
| **Pure error (degrees of freedom)** | | *0.16 (3)* | 0.92 (3) | | | | |
| **Total SS (degrees of freedom)** | | *36.85 (15)* | 32.58 (15) | | | | |

[a] Significant terms (*p*-value≤0.05)

**Table S11. Predicted and observed extraction yields of shrimp body at potential optimal experimental combinations.**

| *UAE* | Extraction time (min) | US power (W) | Solvent/material ratio (mL $g^{-1}$) | Predicted extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) | Experimental extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev) |
|---|---|---|---|---|---|
| **Run A** | 5 | 600 | 10 | 5.84 | 6.73(±0.56)[a] |
| **Run B** | 6 | 600 | 22 | 5.24 | 5.52(±0.45) |
| **Run C** | 16 | 540 | 20 | 5.04 | 4.64(±0.27) |
| ***MAE*** | **Extraction time (min)** | **MW power (W)** | **Solvent/material ratio (mL $g^{-1}$)** | **Predicted extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample)** | **Experimental extraction yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev)** |
| **Run A** | 7 | 30 | 20 | 10.10 | 13.3(±1.1)[a] |
| **Run B** | 9 | 30 | 25 | 8.92 | 9.28 (±0.41) |
| **Run C** | 5 | 30 | 15 | 11.26 | 12.36(±0.33) |

*[a]: Optimal UAE and MAE experimental combinations

**Table S12. Analytical figures of merit of LC-MS/MS for (a) apricots pulp (b) egg yolk (c) shrimp body and (d) shrimp head.**

**(a)**

| Analytical figures of merit | UAE | | | MAE | | |
|---|---|---|---|---|---|---|
| | b-Carotene | Zeaxanthin | Lutein | b-Carotene | Zeaxanthin | Lutein |
| Concentration range ($\mu g\ mL^{-1}$) | 0.5-15 | 5-15 | 0.5-15 | 2.5-20 | 0.5-15 | 0.5-15 |
| Slope (a) ($\pm s_a$) | 0.84 (±0.13) | 0.367 (±0.070) | 0.59 (±0.10) | 0.633 (±0.093) | 1.1 (±0.17) | 0.751 (±0.049) |
| Intercept (b) ($\pm s_b$) | 1.0 (±1.1) | -0.75 (±0.74) | -0.47 (±0.84) | 0.1 (±1.1) | -0.2 (±1.5) | -0.54 (±0.41) |
| LOD ($\mu g\ mL^{-1}$) | 0.75 | 1.59 | 0.19 | 0.85 | 0.40 | 0.25 |
| LOQ ($\mu g\ mL^{-1}$) | 2.28 | 5.31 | 0.63 | 2.58 | 1.21 | 0.75 |
| LOD/LOQ concentration range ($\mu g\ mL^{-1}$) | 0.25-1.25 | 1.0-7.5 | 0.25-1.25 | 0.75-2.7 | 0.25-1.25 | 0.5-1.5 |
| $R^2$ | 0.931 | 0.901 | 0.920 | 0.939 | 0.928 | 0.987 |
| Matrix effect (ME) (%) | 219.32 | 198.92 | 77.44 | 165.27 | 253.20 | 230.00 |

**(b)**

| Analytical figures of merit | UAE | | MAE | |
|---|---|---|---|---|
| | **Lutein** | **Zeaxanthin** | **Lutein** | **Zeaxanthin** |
| **Concentration range ($\mu g\ mL^{-1}$)** | 1-15 | 7.5-20 | 0.5-15 | 0.5-15 |
| **Slope (a) ($\pm s_a$)** | 0.68 (±0.11) | 0.351 (±0.024) | 2.15 (±0.27) | 0.92 (±0.15) |
| **Intercept (b) ($\pm s_b$)** | -0.9(±1.0) | -0.26 (±0.32) | 6.0 (±2.3) | 0.6 (±1.3) |
| **LOD ($\mu g\ mL^{-1}$)** | 0.30 | 2.57 | 0.48 | 0.30 |
| **LOQ ($\mu g\ mL^{-1}$)** | 1.01 | 8.58 | 1.62 | 1.02 |
| **LOD/LOQ concentration range ($\mu g\ mL^{-1}$)** | 0.8-1.8 | 7.5-9.5 | 0.4-1.1 | 0.4-1.1 |
| **$R^2$** | 0.921 | 0.986 | 0.953 | 0.925 |
| **Matrix effect (ME) (%)** | 229.26 | 74.07 | 724.88 | 194.13 |

**(c)**

| Analytical figures of merit | UAE | | | MAE | | |
|---|---|---|---|---|---|---|
| | **Astaxanthin** | **Canthaxantin** | **Zeaxanthin** | **Astaxanthin** | **Canthaxantin** | **Zeaxanthin** |
| **Concentration range ($\mu g\ mL^{-1}$)** | 0.5-15 | 0.5-15 | 0.5-15 | 0.5-15 | 0.5-15 | 0.5-15 |
| **Slope (a) ($\pm s_a$)** | 2.21 (±0.28) | 0.564 (±0.043) | 0.233 (±0.032) | 2.71 (±0.36) | 1.18 (±0.14) | 0.453 (±0.067) |
| **Intercept (b) ($\pm s_b$)** | 2.8 (±2.4) | -0.10 (±0.36) | 0.69 (±0.27) | 21.1 (±3.0) | 5.7 (±1.1) | -0.21 (±0.56) |
| **LOD ($\mu g\ mL^{-1}$)** | 0.32 | 0.37 | 0.20 | 0.25 | 0.35 | 0.40 |
| **LOQ ($\mu g\ mL^{-1}$)** | 0.98 | 1.12 | 0.61 | 0.77 | 1.06 | 1.22 |
| **LOD/LOQ concentration range ($\mu g\ mL^{-1}$)** | 0.25-1.25 | 0.25-1.25 | 0.1-1.0 | 0.1-1.0 | 0.1-1.0 | 0.25-1.25 |
| **$R^2$** | 0.953 | 0.983 | 0.945 | 0.951 | 0.962 | 0.937 |
| **Matrix effect (ME) (%)** | 133.49 | 43.11 | 78.55 | 163.68 | 95.59 | 90.19 |

**(d)**

| Analytical figures of merit | UAE | | | MAE | | |
|---|---|---|---|---|---|---|
| | **Astaxanthin** | **Canthaxantin** | **Zeaxanthin** | **Astaxanthin** | **Canthaxantin** | **Zeaxanthin** |
| **Concentration range ($\mu g\ mL^{-1}$)** | 0.5-15 | 0.5-15 | 0.5-15 | 0.5-15 | 0.5-15 | 0.5-15 |
| **Slope (a) ($\pm s_a$)** | 0.600 (±0.084) | 0.349 (±0.020) | 0.873 (±0.090) | 0.88 (±0.16) | 0.302 (±0.047) | 0.0332 (±0.0055) |
| **Intercept (b) ($\pm s_b$)** | 4.97 (±0.70) | 0.29 (±0.17) | -0.55 (±0.76) | -0.6 (±1.4) | 0.51 (±0.39) | 0.172 (±0.046) |
| **LOD ($\mu g\ mL^{-1}$)** | 0.48 | 0.20 | 0.46 | 0.20 | 0.50 | 0.57 |
| **LOQ ($\mu g\ mL^{-1}$)** | 1.46 | 0.62 | 1.39 | 0.62 | 1.52 | 1.72 |
| **LOD/LOQ concentration range ($\mu g\ mL^{-1}$)** | 0.5-1.5 | 0.15-1.0 | 0.25-1.25 | 0.25-1.25 | 0.5-1.5 | 0.5-1.5 |
| **$R^2$** | 0.945 | 0.990 | 0.970 | 0.906 | 0.933 | 0.924 |
| **Matrix effect (ME) (%)** | 133.49 | 43.11 | 78.55 | 53.15 | 2.54 | 63.73 |

**Table S13. Method validation results of LC-MS/MS for (a) apricots pulp (b) egg yolk (c) shrimp body and (d) shrimp head.**

**(a)**

| Carotenoids | UAE QC levels | | |
|---|---|---|---|
| ***b-Carotene*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 10.82 | 10.42 | 7.21 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 15.05 | 9.80 | 6.69 |
| **Accuracy** | 83.00 | 106.00 | 94.53 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 119.56 | |
| ***Zeaxanthin*** | **7.5µg mL$^{-1}$ (n=3)** | **10.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 12.80 | 8.66 | 7.34 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 12.01 | 7.77 | 6.53 |
| **Accuracy** | 109.80 | 79.2 | 104.60 |
| **Process recovery at spike level 10.0 µg mL$^{-1}$** | | 67.92 | |
| ***Lutein*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 10.81 | 9.96 | 6.54 |
| **Inter-day precision (RSD$_r$%), N=3** | 14.69 | 7.10 | 5.18 |
| **Accuracy** | 104.00 | 96.40 | 109.73 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 38.51 | |
| **Carotenoids** | **MAE QC levels** | | |
| ***b-Carotene*** | **5.0µg mL$^{-1}$ (n=3)** | **10.0 µg mL$^{-1}$ (n=3)** | **15.0 µg mL$^{-1}$(n=3)** |
| **Intra-day precision (RSD$_r$%)** | 8.45 | 4.86 | 7.31 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 9.19 | 4.25 | 5.79 |
| **Accuracy** | 86.80 | 89.80 | 86.70 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 97.91 | |
| ***Zeaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$(n=3)** |
| **Intra-day precision (RSD$_r$%)** | 14.14 | 2.23 | 4.39 |
| **Inter-day precision (RSD$_r$%), N=3** | 11.67 | 8.91 | 10.95 |
| **Accuracy** | 100.80 | 87.80 | 86.60 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 93.77 | |
| ***Lutein*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$(n=3)** |
| **Intra-day precision (RSD$_r$%)** | 9.92 | 9.78 | 4.91 |
| **Inter-day precision (RSD$_r$%), N=3** | 11.61 | 9.92 | 11.98 |
| **Accuracy** | 106.60 | 108.20 | 101.40 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 116.71 | |

[a]*n: the number of QC replicates and N: the number of consecutive days required for inter-day precision determination*

**(b)**

| Carotenoids | UAE QC levels | | |
|---|---|---|---|
| ***Lutein*** | **2.5µg mL$^{-1}$(n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **10.0 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 9.37 | 9.76 | 6.62 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 10.20 | 9.67 | 4.74 |
| **Accuracy** | 110.0 | 83.35 | 97.24 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 72.99 | |
| ***Zeaxanthin*** | **9.0µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** | **18.0 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 1.83 | 6.04 | 2.62 |
| **Inter-day precision (RSD$_r$%), N=3** | 9.12 | 4.28 | 1.92 |
| **Accuracy** | 97.52 | 105.44 | 104.49 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 62.96 | |
| **Carotenoids** | **MAE QC levels** | | |
| ***Lutein*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 13.41 | 4.54 | 1.37 |
| **Inter-day precision (RSD$_r$%), N=3** | 14.84 | 4.99 | 2.06 |
| **Accuracy** | 108.00 | 99.8 | 108.3 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 75.45 | |
| ***Zeaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$(n=3)** |
| **Intra-day precision (RSD$_r$%)** | 14.27 | 9.27 | 2.11 |
| **Inter-day precision (RSD$_r$%), N=3** | 11.41 | 8.43 | 6.42 |
| **Accuracy** | 84.0 | 100.06 | 98.3 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 64.74 | |

[a]*n: the number of QC replicates and N: the number of consecutive days required for inter-day precision determination*

**(c)**

| Carotenoids | UAE QC levels | | |
|---|---|---|---|
| ***Astaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 3.84 | 12.94 | 3.83 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 3.60 | 9.16 | 12.75 |
| **Accuracy** | 82.0 | 89.0 | 82.7 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 77.01 | |
| ***Canthaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 12.17 | 10.66 | 14.35 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 8.86 | 11.86 | 11.62 |
| **Accuracy** | 92.0 | 110.0 | 108.9 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 48.94 | |
| ***Zeaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 14.42 | 12.16 | 12.78 |
| **Inter-day precision (RSD$_r$%), N=3** | 11.21 | 8.95 | 13.56 |
| **Accuracy** | 89.0 | 84.4 | 109.1 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 66.06 | |
| **Carotenoids** | **MAE QC levels** | | |
| ***Astaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 6.64 | 0.48 | 2.13 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 5.89 | 1.00 | 3.47 |
| **Accuracy** | 107.0 | 109.0 | 89.3 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 93.41 | |
| ***Canthaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 14.94 | 6.71 | 4.15 |
| **Inter-day precision (RSD$_r$%), N=3** | 10.77 | 6.22 | 8.69 |
| **Accuracy** | 110.0 | 81.0 | 100.1 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 54.18 | |
| ***Zeaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 0.30 | 0.20 | 14.61 |
| **Inter-day precision (RSD$_r$%), N=3** | 10.40 | 13.41 | 12.17 |
| **Accuracy** | 110.0 | 81.6 | 109.3 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 80.61 | |

[a]*n: the number of QC replicates and N: the number of consecutive days required for inter-day precision determination*

**(d)**

| Carotenoids | UAE QC levels | | |
|---|---|---|---|
| ***Astaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 8.60 | 10.01 | 3.18 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 12.38 | 7.30 | 6.53 |
| **Accuracy** | 90.0 | 99.2 | 87.73 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 85.85 | |
| ***Canthaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 14.57 | 7.73 | 8.48 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 11.68 | 5.58 | 14.36 |
| **Accuracy** | 102.00 | 83.80 | 95.67 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 57.38 | |
| ***Zeaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 14.20 | 9.07 | 1.34 |
| **Inter-day precision (RSD$_r$%), N=3** | 12.80 | 6.78 | 8.47 |
| **Accuracy** | 108.0 | 89.0 | 108.00 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 72.17 | |
| **Carotenoids** | **MAE QC levels** | | |
| ***Astaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 10.99 | 1.88 | 4.43 |
| **Inter-day precision (RSD$_r$%), N=3[a]** | 8.99 | 5.58 | 11.60 |
| **Accuracy** | 98.0 | 102.2 | 109.9 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 91.71 | |
| ***Canthaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 5.24 | 4.54 | 5.85 |
| **Inter-day precision (RSD$_r$%), N=3** | 11.64 | 11.49 | 10.12 |
| **Accuracy** | 110.0 | 98.4 | 105.2 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 63.81 | |
| ***Zeaxanthin*** | **1.0µg mL$^{-1}$ (n=3)** | **5.0 µg mL$^{-1}$ (n=3)** | **12.5 µg mL$^{-1}$ (n=3)** |
| **Intra-day precision (RSD$_r$%)** | 4.52 | 13.72 | 0.91 |
| **Inter-day precision (RSD$_r$%), N=3** | 6.21 | 10.93 | 7.10 |
| **Accuracy** | 108.0 | 110.4 | 95.2 |
| **Process recovery at spike level 5.0 µg mL$^{-1}$** | | 67.10 | |

[a]*n: the number of QC replicates and N: the number of consecutive days required for inter-day precision determination*

**Table S14. Carotenoid content measured by LC-MS/MS for the apricot samples used in NMR analysis.**

| Extract | Carotenoid yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev), n=3[a] | Extract | Carotenoid yield (mg of carotenoids 100 $g^{-1}$ dry sample) (±stdev), n=3[a] |
|---|---|---|---|
| MAE_$CHCl_3$-MeOH_2:1 | 3.30(±0.35) | UAE_EtOH | 5.03(±0.31) |
| MAE_$CHCl_3$ | 1.192(±0.095) | UAE_$CHCl_3$-MeOH_1:1 | 9.39(±0.15) |
| MAE_Acetone | 0.48(±0.26) | UAE_Run 3_Two level design | 9.81(±0.21) |
| MAE_*n*-Hexane-Acetone | 0.84(±0.31) | UAE_Run 7_Two level design | 10.17(±0.33) |
| MAE_*n*-Hexane-Acetone-EtOH 2:1:1 | 0.27(±0.15) | UAE_Run 13_BBD | 13.72(±0.59) |
| UAE_$CHCl_3$-MeOH_2:1 | 0.94(±0.092) | UAE_Optimal values | 11.21(±0.34) |
| UAE_$CHCl_3$ | 0.34(±0.10) | MAE_Run 2_Two level design | 20.33(±0.70) |
| UAE_Acetone | 0.75(±0.17) | MAE_Run 3_Two level design | 19.861(±0.096) |
| UAE_*n*-Hexane-Acetone | 2.66(±0.20) | MAE_Run 8_Two level design | 16.38(±0.80) |
| UAE_*n*-Hexane-Acetone-EtOH 2:1:1 | 2.020(±0.067) | MAE_Run 6_BBD | 24.30(±0.72) |
| UAE_Run 6_BBD | 4.01(±0.17) | MAE_Run 9_BBD | 21.09(±0.68) |
| UAE_Run 11_BBD | 1.79(±0.49) | MAE_Run 12_BBD | 20.44(±0.55) |
| Folch | 1.52(±14) | UAE_Run 4_Two level design | 16.15(±0.20) |
| MAE_$CHCl_3$-MeOH_1:1 | 6.97(±0.27) | UAE_Run 8_Two level design | 18.82(±0.29) |
| MAE_EtOH-Acetone | 10.61(±0.86) | MAE_Optimal values | 19.28(±0.27) |
| MAE_EtOH | 11.09(±0.98) | | |
| UAE_EtOH-Acetone | 6.13(±0.23) | | |

*a:number of replicates*

# REFERENCES


[1] G. M. Cragg and D. J. Newman, Natural products: A continuing source of novel drug leads, *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1830, no. 6, 2013, pp. 3670–3695.

[2] J. Azmir *et al.*, Techniques for extraction of bioactive compounds from plant materials: A review, *J. Food Eng.*, vol. 117, no. 4, 2013, pp. 426–436.

[3] A. L. Harvey, Natural products in drug discovery, *Drug Discov. Today*, vol. 13, no. 19–20, 2008, pp. 894–901.

[4] D. J. Newman and G. M. Cragg, Natural products as sources of new drugs from 1981 to 2014, *J. Nat. Prod.*, vol. 79, no. 3, 2016, pp. 629–661.

[5] A. G. Atanasov *et al.*, Discovery and resupply of pharmacologically active plant-derived natural products: A review, *Biotechnol. Adv.*, vol. 33, no. 8, 2015, pp. 1582–1614.

[6] R. K. Saini, S. H. Moon, and Y. S. Keum, An updated review on use of tomato pomace and crustacean processing waste to recover commercially vital carotenoids, *Food Res. Int.*, vol. 108, 2018, pp. 516–529.

[7] W. T. Lai *et al.*, A review: Modified agricultural by-products for the development and fortification of food products and nutraceuticals, *Trends Food Sci. Technol.*, vol. 59, 2017, pp. 148–160.

[8] F. Chemat, N. Rombaut, A. G. Sicaire, A. Meullemiestre, A. S. Fabiano-Tixier, and M. Abert-Vian, Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review, *Ultrason. Sonochem.*, vol. 34, 2017, pp. 540–560,.

[9] V. G. Zuin and L. Z. Ramin, Green and Sustainable Separation of Natural Products from agro-Industrial waste: Challenges, potentialities, and perspectives on emerging approaches, *Top. Curr. Chem.*, vol. 376, no. 1, 2018, pp. 1–54.

[10] M. N. Shashirekha, S. E. Mallikarjuna, and S. Rajarathnam, Status of Bioactive compounds in foods, with focus on fruits and vegetables, *Crit. Rev. Food Sci. Nutr.*, vol. 55, no. 10, 2015, pp. 1324–1339.

[11] A. Valdés, A. C. Mellinas, M. Ramos, N. Burgos, A. Jiménez, and M. C. Garrigós, Use of herbs, spices and their bioactive compounds in active food packaging, *RSC Adv.*, vol. 5, no. 50, 2015, pp. 40324–40335.

[12] G. González-Aguilar, R. M. Robles-Sánchez, M. A. Martínez-Téllez, G. I. Olivas, E. Alvarez-Parrilla, and L. A. De La Rosa, Bioactive compounds in fruits: Health benefits and effect of storage conditions, *Stewart Postharvest Rev.*, vol. 4, no. 3, 2008, pp. 1-10.

[13] P. Kaushik *et al.*, Breeding vegetables with increased content in bioactive phenolic acids, *Molecules*, vol. 20, no. 10, 2015, pp. 18464–18481.

[14] S. S. Smuda, S. M. Mohsen, K. Olsen, and M. H. Aly, Bioactive compounds and antioxidant activities of some cereal milling by-products, *J. Food Sci. Technol.*, vol. 55, no. 3, 2018, pp. 1134–1142.

[15] I. Hamed, F. Özogul, Y. Özogul, and J. M. Regenstein, Marine Bioactive Compounds and Their Health Benefits: A Review, *Compr. Rev. Food Sci. Food Saf.*, vol. 14, no. 4, 2015, .pp. 446–465.

[16] M. E. Valverde, T. Hernández-Pérez, and O. Paredes-López, Edible Mushrooms: Improving Human Health and Promoting Quality Life, *Int. J. Microbiol.*, vol., 2015, pp. 1–14,.

[17] K. T. Khaw *et al.*, Randomised trial of coconut oil, olive oil or butter on blood lipids and other cardiovascular risk factors in healthy men and women, *BMJ Open*, vol. 8, no. 3, 2018, pp. 1–14.

[18] R. K. Saini, S. H. Nile, and S. W. Park, Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities, *Food Res. Int.*, vol. 76, 2015, pp. 735–750.

[19] A. M. Goula and H. N. Lazarides, Integrated processes can turn industrial food waste into valuable food by-products and/or ingredients: The cases of olive mill and pomegranate wastes, *J. Food Eng.*, vol. 167, 2015, pp. 45–50.

[20] A. L. Skaltsounis, A. Argyropoulou, N. Aligiannis, and N. Xynos, *Recovery of High Added Value Compounds from Olive Tree Products and Olive Processing Byproducts*. AOCS Press, 2015.

[21] M. A. Bustamante, R. Moral, C. Paredes, A. Pérez-Espinosa, J. Moreno-Caselles, and M. D. Pérez-Murcia, Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry, *Waste Manag.*, vol. 28, no. 2, 2008, pp. 372–380.

[22] A. Teixeira *et al.*, Natural bioactive compounds from winery by-products as health promoters: A review, *Int. J. Mol. Sci.*, vol. 15, no. 9, 2014, pp. 15638–15678.

[23] I. Jantan, W. Ahmad, and S. N. A. Bukhari, Corrigendum: Plant-derived immunomodulators: an insight on their preclinical evaluation and clinical trials, *Front. Plant Sci.*, vol. 9, 2018, pp. 1–18.

[24] K. de la Caba *et al.*, From seafood waste to active seafood packaging: An emerging opportunity of the circular economy, *J. Clean. Prod.*, vol. 208, 2019, pp. 86–98.

[25] S. A. Mehlenbacher, V. Cociu and F. L. Hough, Apricots (*Prunus*), *Acta Hortic.*, vol. 290, 1991, pp. 65-110.

[26] F. Fratianni, M. N. Ombra, A. d'Acierno, L. Cipriano, and F. Nazzaro, Apricots: biochemistry and functional properties, *Curr. Opin. Food Sci.*, vol. 19, 2018, pp. 23–29,.

[27] S. G. Rudra, N. Jakhar, C. Kaur, and J. Nishad, Food Industry waste: mine of nutraceuticals, *Int. J. Sci. Enviroment Technol.*, vol. 4, no. 1, 2015, pp. 205–229.

[28] D. Cheaib, N. El Darra, H. N. Rajha, I. El Ghazzawi, R. G. Maroun, and N. Louka, Biological activity of apricot byproducts polyphenols using solid–liquid and infrared-assisted technology, *J. Food Biochem.*, vol. 42, no. 5, 2018, pp. 1–9,.

[29] O. E. Campbell and O. I. Padilla-Zakour, Phenolic and carotenoid composition of canned peaches (*Prunus persica*) and apricots (*Prunus armeniaca*) as affected by variety and peeling, *Food Res. Int.*, vol. 54, no. 1, 2013, pp. 448–455,.

[30] D. Ruiz, J. Egea, F. A. Tomás-Barberán and M. I. Gil, Carotenoids from new apricot (*Prunus armeniaca* L.) varieties and their relationship with flesh and skin Color, *J. Agric. Food Chem.*, vol. 53, no. 16, 2005, pp. 6368–6374.

[31] P. Sriket, S. Benjakul, W. Visessanguan, and K. Kijroongrojana, Comparative studies on chemical composition and thermal properties of black tiger shrimp (*Penaeus monodon*) and white shrimp (*Penaeus vannamei*) meats, *Food Chem.*, vol. 103, no. 4, 2007, pp. 1199–1207.

[32] P. Kandra, M. M. Challa, and H. Kalangi Padma Jyothi, Efficient use of shrimp waste: Present and future trends, *Appl. Microbiol. Biotechnol.*, vol. 93, no. 1, 2012, pp. 17–29.

[33] M. López, L. Arce, J. Garrido, A. Ríos, and M. Valcárcel, Selective extraction of astaxanthin from crustaceans by use of supercritical carbon dioxide, *Talanta*, vol. 64, no. 3, 2004, pp. 726–731.

[34] N. Mezzomo, E. de Paz, M. Maraschin, Á. Martín, M. J. Cocero, and S. R. S. Ferreira, Supercritical anti-solvent precipitation of carotenoid fraction from pink shrimp residue: Effect of operational conditions on encapsulation efficiency, *J. Supercrit. Fluids*, vol. 66, 2012, pp. 342–349.

[35] I. Seuss-Baum and F. Nau, The nutritional quality of eggs, *Improving the Safety and Quality of Eggs and Egg Products*, F. Van Immerseel et al, eds, Woodhead Publishing Limited, 2011, pp. 201-236.

[36] M. Mahmoudi and M. A. Ebrahimzadeh, Anti-inflammatory and analgesic effects of egg yolk : a comparison between organic and machine made, *Eur. Rev. Med. Pharmaacological Sci.*, vol. 17, 2013, pp. 472–476.

[37] K. Mitri, R. Shegokar, S. Gohla, C. Anselmi, and R. H. Müller, Lipid nanocarriers for dermal delivery of lutein : Preparation, characterization, stability and performance, *Int. J. Pharm.*, vol. 414, no. 1–2, 2011, pp. 267–275.

[38] S. Shanmugam *et al.*, Enhanced bioavailability and retinal accumulation of lutein from self-emulsifying phospholipid suspension (SEPS), *Int. J. Pharm.*, vol. 412, no. 1–2, 2011, pp. 99–105.

[39] J. Berg and D. Lin, “Lutein and Zeaxanthin : An overview of metabolism and eye health,” vol. 2, 2014, pp. 4–5.

[40] K. Zaheer, Hen egg carotenoids (lutein and zeaxanthin) and nutritional impacts on human health: a review, *CyTA - J. Food*, vol. 15, no. 3, 2017, pp. 474–487,.

[41] C. Nimalaratne, J. Wu, and A. Schieber, Egg yolk carotenoids: Composition, analysis, and effects of processing on their stability, *Carotenoid Cleavage Products*, P. Winterhalter et al, eds, American Chemical Society, 2013, pp. 219–225.

[42] M. I. Fernández-Mar, R. Mateos, M. C. García-Parrilla, B. Puertas, and E. Cantos-Villar, Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review, *Food Chem.*, vol. 130, no. 4, 2012, pp. 797–813.

[43] M. Plaza and C. Turner, Pressurized hot water extraction of bioactives, *TrAC - Trends Anal. Chem.*, vol. 71, 2015, pp. 39–54.

[44] F. Chemat, M. A. Vian, and G. Cravotto, Green extraction of natural products: Concept and principles, *Int. J. Mol. Sci.*, vol. 13, no. 7, 2012, pp. 8615–8627,.

[45] H. Wijngaard, M. B. Hossain, D. K. Rai, and N. Brunton, Techniques to extract bioactive compounds from food by-products of plant origin, *Food Res. Int.*, vol. 46, no. 2, 2012, pp. 505–513.

[46] A. Ghasemzadeh and N. Ghasemzadeh, Flavonoids and phenolic acids: Role and biochemical activity in plants and human, *J. Med. Plants Res.*, vol. 5, no. 31, 2011, pp. 6697–6703.

[47] K. T. Amorim-Carrilho, A. Cepeda, C. Fente, and P. Regal, Review of methods for analysis of carotenoids, *TrAC - Trends Anal. Chem.*, vol. 56, 2014, pp. 49–73.

[48] S. Cicerale, L. Lucas, and R. Keast, Biological activities of phenolic compounds present in virgin olive oil, *Int. J. Mol. Sci.*, vol. 11, no. 2, 2010, pp. 458–479.

[49] S. Vats, Methods for extractions of value-added nutraceuticals from lignocellulosic wastes and their health application, *Ingredients Extraction by Physicochemical Methods in Food,* P. Grumezescu et al, eds, Elsevier, 2017, pp. 1-64.

[50] F. Bakkali, S. Averbeck, D. Averbeck, and M. Idaomar, Biological effects of essential oils - A review, *Food Chem. Toxicol.*, vol. 46, no. 2, 2008, pp. 446–475.

[51] M.H. Abd El-Salam and S. El-Shibiny, Separation of bioactive whey proteins and peptides, *Ingredients Extraction by Physicochemical Methods in Food,* P. Grumezescu et al, eds, Elsevier, 2017, pp. 464-519.

[52] I.I. Muhamad, N.D. Hassan, S.N.H. Mamat, N.M. Nawi, W.A. Rashid and N.A Tan, Extraction technologies and solvents of phytocompounds from plant materials: Physicochemical characterization and identification of ingredients and bioactive compounds from plant extract using various instrumentations, *Ingredients Extraction by Physicochemical Methods in Food,* P. Grumezescu et al, eds, Elsevier, 2017, pp. 548-584.

[53] C. S. Boon, D. J. McClements, J. Weiss, and E. A. Decker, Factors influencing the chemical stability of carotenoids in foods, *Crit. Rev. Food Sci. Nutr.*, vol. 50, no. 6, 2010, pp. 515–532.

[54] I. Strati *et al.*, Carotenoids from foods of plant, animal and marine origin: An efficient HPLC-DAD separation method," *Foods*, vol. 1, no. 1, 2012, pp. 52–65.

[55] A. Singh, S. Ahmad, and A. Ahmad, Applications of carotenoids-a review, *RSC Adv.*, vol. 5, 2015, pp. 62358–62393.

[56] E.-S. Abdel-Aal *et al.*, Dietary sources of lutein and zeaxanthin carotenoids and their role in eye health, *Nutrients*, vol. 5, no. 4, 2013, pp. 1169–1185.

[57] E. Christaki, E. Bonos, I. Giannenas, and P. Florou-Paneri, Functional properties of carotenoids originating from algae, *J. Sci. Food Agric.*, vol. 93, no. 1, 2013, pp. 5–11.

[58] A. Schieber and R. Carle, Occurrence of carotenoid cis-isomers in food: Technological, analytical, and nutritional implications, *Trends Food Sci. Technol.*, vol. 16, no. 9, 2005, pp. 416–422.

[59] G. Riccioni, N. D' Orazio, C. Salvatore, S. Franceschelli, M. Pesce, and L. Speranza, Carotenoids and vitamins C and E in the prevention of

cardiovascular disease, *Int. J. Vitam. Nutr. Res.*, vol. 82, no. 1, 2012, pp. 15–26.

[60] R. G. Fassett and J. S. Coombes, Astaxanthin: A potential therapeutic agent in cardiovascular disease, *Mar. Drugs*, vol. 9, no. 3, 2011, pp. 447–465.

[61] Y. S. Oh and H.-S. Jun, Role of bioactive food components in diabetes prevention: Effects on beta-cell function and preservation, *Nutr. Metab. Insights*, vol. 7, 2014, pp. 51-59.

[62] J.P. Yuan, J. Peng, K. Yin, and J.-H. Wang, Potential health-promoting effects of astaxanthin: A high-value carotenoid mostly from microalgae, *Mol. Nutr. Food Res.*, vol. 55, no. 1, 2011, pp. 150–165.

[63] M. Chatterjee, K. Roy, M. Janarthan, S. Das, and M. Chatterjee, Biological activity of carotenoids: Its implications in cancer risk and prevention, *Curr. Pharm. Biotechnol.*, vol. 13, no. 1, 2012, pp. 180–190.

[64] T. Tanaka, M. Shnimizu, H. Moriwaki, T. Tanaka, M. Shnimizu, and H. Moriwaki, Cancer chemoprevention by carotenoids, *Molecules*, vol. 17, no. 3, 2012, pp. 3202–3242.

[65] J. M. Barbosa-Filho *et al.*, Sources of alpha-, beta-, gamma-, delta- and epsilon-carotenes: A twentieth century review, *Brazilian J. Pharmacogn.*, vol. 18, no. 1, 2008, pp. 135–154.

[66] R. W. S. Chung, P. Leanderson, A. K. Lundberg, and L. Jonasson, Lutein exerts anti-inflammatory effects in patients with coronary artery disease, *Atherosclerosis*, vol. 262, 2017, pp. 87–93.

[67] T. P. Anunciato and P. Alves, Carotenoids and polyphenols in nutricosmetics , nutraceuticals , and cosmeceuticals,*J. Cosmet. Dermatol.*, vol. 11, 2012, pp. 51–54,.

[68] E. Bovier and B. Hammond, The macular carotenoids lutein and zeaxanthin are related to increased bone density in young healthy adults, *Foods*, vol. 6, no. 9, 2017, p. 78-86.

[69] V. Juturu and O. Health, Meso-Zeaxanthin (MZ): Current perspectives and new insights meso-zeaxanthin, *Adv. Ophthalmol. Vis. Syst.*, vol.1, no.2, 2014, pp. 00010.

[70] T. Madaan *et al.*, Lutein, a versatile phyto-nutraceutical: An insight on pharmacology, therapeutic indications, challenges and recent advances in drug delivery, *Pharma Nutrition*, vol. 5, no. 2, 2017, pp. 64–75.

[71] P. Chávez-Crooker, J. Obreque, J. Vera, and K. Moya,Role of astaxanthin in cosmeceutical applications, *Marine Cosmeceuticals: Trends and Prospects,* S.K. Kim, ed, Taylor&Francis Group, 2011, pp. 119-124.

[72] T. Esatbeyoglu and G. Rimbach, Canthaxanthin: From molecule to function, *Mol. Nutr. Food Res.*, vol. 61, no. 6, 2017, pp. 1–49.

[73] T. Tsiaka, V.J. Sinanoglou and P. Zoumpoulakis, Extracting bioactive compounds from natural sources using green high-energy approaches: Trends and opportunities in lab- and large-scale applications, *Ingredients Extraction by Physicochemical Methods in Food,* P. Grumezescu et al, eds, Elsevier, 2017, pp. 332-390.

[74] H. H. Jeleń, M. Majcher, and M. Dziadas, Microextraction techniques in the analysis of food flavor compounds: A review, *Anal. Chim. Acta*, vol. 738, 2012, pp. 13–26.

[75] A. K. Pabby and A. M. Sastre, State-of-the-art review on hollow fibre contactor technology and membrane-based extraction processes, *J. Memb. Sci.*, vol. 430, 2013, pp. 263–303.

[76] P. Viñas, N. Campillo, I. López-García, and M. Hernández-Córdoba, Dispersive liquid-liquid microextraction in food analysis. A critical review, *Anal. Bioanal. Chem.*, vol. 406, no.8, 2013, pp. 2067-2099.

[77] G. Joana Gil-Chávez *et al.*, Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients: An overview," *Compr. Rev. Food Sci. Food Saf.* vol. 12, no. 1, 2013, pp. 5–23.

[78] L. Wang and C. L. Weller, Recent advances in extraction of nutraceuticals from plants, *Trends Food Sci. Technol.*, vol. 17, no. 6, 2006, pp. 300–312,.

[79] H. H. Wijngaard, O. Trifunovic, and P. Bongers, Novel extraction techniques for phytochemicals, *Handbook of Plant Food Phytochemicals:*

*Sources, Stabilty and Extraction*,B.K. Tiwari et al, eds, Wiley, 2013, pp. 412–433.

[80] F. G. C. Ekezie, D. W. Sun, and J. H. Cheng, Acceleration of microwave-assisted extraction processes of food components by integrating technologies and applying emerging solvents: A review of latest developments, *Trends Food Sci. Technol.*, vol. 67, 2017, pp. 160–172.

[81] S. Armenta, S. Garrigues, and M. de la Guardia, Green analytical chemistry, *TrAC-Trends Anal. Chem.*, vol. 27, no. 6, 2008, pp. 497-511.

[82] L. Moity, M. Durand, A. Benazzouz, V. Molinier and J.M. Aubry, In silico search for alternative green solvents, *Alternative Solvents for Natural Products Extraction*,F. Chemat et al, eds, Springer, 2014, pp. 1-24.

[83] A. C. Soria and M. Villamiel, Effect of ultrasound on the technological properties and bioactivity of food: A review, *Trends Food Sci. Technol.*, vol. 21, no. 7, 2010, pp. 323–331.

[84] Y. Picó, Ultrasound-assisted extraction for food and environmental samples, *TrAC-Trends Anal. Chem.*, vol. 43, 2013, pp. 84–99.

[85] J. Chandrapala, C. M. Oliver, S. Kentish, and M. Ashokkumar, Use of power ultrasound to improve extraction and modify phase transitions in food processing, *Food Rev. Int.*, vol. 29, no. 1, 2013, pp. 67–91.

[86] T. Jain, V. Jain, and R. Pandey, Microwave assisted extraction for phytoconstituents-An overview, *Asian J. Res. Chem.*, vol. 2, no. 1, 2009, pp. 19–25.

[87] P.C. Veggi, J. Martinez, and M.A.A. Meireles, Fundamentals of microwave extraction, *Microwave-Assisted Extraction for Bioactive Compounds: Theory and Practice*,F. Chemat et al, eds, Springer, 2013, pp. 15-52.

[88] P. Tatke, and Y. Jaiswal, An overview of MAE and its applications in herbal drug research, *Res. J. Med. Plant*, vol. 5, no. 1, 2009, pp. 21-31.

[89] Y. Li, A. S. Fabiano-Tixier, M. A. Vian, and F. Chemat, Solvent-free microwave extraction of bioactive compounds provides a tool for green analytical chemistry, *TrAC-Trends Anal. Chem.*, vol. 47, 2013, pp. 1–11.

[90] M. Abert-Vian, T. Allaf, E. Vorobiev, and F. Chemat, Solvent-free extraction: myth or reality? *Alternative Solvents for Natural Products Extraction*,F. Chemat et al, eds, Springer, 2014, pp. 25-38.

[91] Zill-E-Huma, M. Abert-Vian, M. Elmaataoui, and F. Chemat, A novel idea in food extraction field: Study of vacuum microwave hydrodiffusion technique for by-products extraction, *J. Food Eng.*, vol. 105, no. 2, 2011, pp. 351–360.

[92] P. Sun and D. W. Armstrong, Ionic liquids in analytical chemistry, *Anal. Chim. Acta*, vol. 661, no. 1, 2010, pp. 1–16.

[93] N. Gjineci, E. Boli, A. Tzani, A. Detsi, and E. Voutsas, Separation of the ethanol/water azeotropic mixture using ionic liquids and deep eutectic solvents,*Fluid Phase Equilib.*, vol. 424, 2016, pp. 1–7.

[94] M. W. Nam *et al.*, Enhanced extraction of bioactive natural products using tailor-made deep eutectic solvents: application to flavonoid extraction from Flos sophorae, *Green Chem.*, vol. 17, no. 3, 2015, pp. 1718–1727.

[95] F. Pena-Pereira and J. Namieśnik, Ionic liquids and deep eutectic mixtures: Sustainable solvents for extraction processes, *Chem Sus. Chem.*, vol. 7, no. 7, 2014, pp. 1784–1800.

[96] Q. Cui *et al.*, Sustainable deep eutectic solvents preparation and their efficiency in extraction and enrichment of main bioactive flavonoids from sea buckthorn leaves, *J Clean Prod.*, vol. 184, 2018, pp. 826-835.

[97] K.M. Jeong *et al.*, Multi-functioning deep eutectic solvents as extraction and storage media for bioactive natural products that are readily applicable to cosmetic products, *J Clean Prod.*, vol. 151, 2017, pp. 87-95.

[98] Y.H. Choi, J. van Spronsen, Y. Dai, M. Verberne, F. Hollmann, I.W. Arends, G.-J. Witkamp, and R.Verpoorte,. Are natural deep eutectic solvents the missinglink in understanding cellular metabolism and physiology?, *Plant Physiol.*, vol. 156, no 4, 2011, pp.1701-1705.

[99] Y. Dai, J. van Spronsen, G.J. Witkamp, R. Verpoorte, and Y.H. Choi,. Natural deep eutectic solvents as new potential media for green technology, *Anal. Chim. Acta*, vol. 766, 2013, pp. 61-68.

[100] M. Vinatoru, T. J. Mason, and I. Calinescu, Ultrasonically assisted extraction (UAE) and microwave assisted extraction (MAE) of functional compounds from plant materials, *TrAC Trends Anal. Chem.*, vol. 97, 2017, pp. 159–178.

[101] T. D. Ho, A. J. Canestraro, and J. L. Anderson, Ionic liquids in solid-phase microextraction : A review, *Anal. Chim. Acta*, vol. 695, no. 1–2, 2011, pp. 18–43.

[102] M. J. Trujillo-Rodríguez, P. Rocío-Bautista, V. Pino, and A. M. Afonso, Ionic liquids in dispersive liquid-liquid microextraction, *TrAC-Trends Anal. Chem.*, vol. 51, 2013, pp. 87–106.

[103] C. Ma, T. Liu, L. Yang, Y. Zu, S. Wang, and R. Zhang, Study on ionic liquid-based ultrasonic-assisted extraction of biphenyl cyclooctene lignans from the fruit of *Schisandra chinensis* Baill, *Anal. Chim. Acta*, vol. 689, no. 1, 2011,pp. 110–116.

[104] G. Zu *et al.*, Ultrasound-assisted extraction of carnosic acid and rosmarinic acid using ionic liquid solution from *Rosmarinus officinalis*, *Int. J. Mol. Sci.*, vol. 13, no. 9, 2012, pp. 11027–11043.

[105] W. Bi, M. Tian, J. Zhou, and K. H. Row, Task-specific ionic liquid-assisted extraction and separation of astaxanthin from shrimp waste, *J. Chromatogr. B*, vol. 878, no. 24, 2010, pp. 2243–2248.

[106] W. Ma, Y. Lu, R. Hu, J. Chen, Z. Zhang, and Y. Pan, Application of ionic liquids based microwave-assisted extraction of three alkaloids N-nornuciferine, O-nornuciferine, and nuciferine from lotus leaf, *Talanta*, vol. 80, no. 3, 2010, pp. 1292–1297.

[107] T. Liu, X. Sui, R. Zhang, L. Yang, Y. Zu, and L. Zhang, Application of ionic liquids based microwave-assisted simultaneous extraction of carnosic acid, rosmarinic acid and essential oil from *Rosmarinus officinalis*, *J. Chromatogr. A*, vol. 1218, no. 47, 2011, pp. 8480–8489.

[108] Z. Wei *et al.*, Ionic liquids-based microwave-assisted extraction of active components from pigeon pea leaves for quantitative analysis, *Sep. Purif. Technol.*, vol. 102, 2013, pp. 75–81.

[109] Z. Lou, H. Wang, S. Zhu, S. Chen, M. Zhang, and Z. Wang, Ionic liquids based simultaneous ultrasonic and microwave assisted extraction of phenolic compounds from burdock leaves, *Anal. Chim. Acta*, vol. 716, 2012, pp. 28–33.

[110] C. Lu, H. Wang, W. Lv, C. Ma, P. Xu, J. Zhu, J. Xie and B. Liu,. Ionic liquid-based ultrasonic/microwave-assisted extraction combined with UPLC for the determination of tannins in *Galla chinensis*, *Nat. Prod. Res.*, vol. 26, no. 19, 2012, pp.1842–1847.

[111] E. Yilmaz and M. Soylak, Ultrasound assisted-deep eutectic solvent extraction of iron from sheep, bovine and chicken liver samples, *Talanta*, vol. 136, 2015, pp. 170–173.

[112] H. Zhang, B. Tang, and K. H. Row, A green deep eutectic solvent-based ultrasound-assisted method to extract astaxanthin from shrimp byproducts," *Anal. Lett.*, vol. 47, no. 5, 2014, pp. 742–749.

[113] M. Cvjetko Bubalo, N. Ćurko, M. Tomašević, K. Kovačević Ganić, and I. Radojcic Redovnikovic, Green extraction of grape skin phenolics by using deep eutectic solvents, *Food Chem.*, vol. 200, 2016, pp. 159–166.

[114] Q. Cui *et al.*, Deep eutectic solvent-based microwave-assisted extraction of genistin, genistein and apigenin from pigeon pea roots, *Sep. Purif. Technol.*, vol. 150, 2015, pp. 63–72.

[115] Z. F. Wei *et al.*, Fast and green extraction and separation of main bioactive flavonoids from *Radix Scutellariae*, *Ind. Crops Prod.*, vol. 63, 2015, pp. 175–181.

[116] T. Zhou, X. Xiao, and G. Li, Microwave accelerated selective Soxhlet extraction for the determination of organophosphorus and carbamate pesticides in ginseng with gas chromatography/mass spectrometry, *Anal. Chem.*, vol. 84, no. 13, 2012, pp. 5816–5822.

[117] M. D. Luque de Castro and F. Priego-Capote, Soxhlet extraction: Past and present panacea, *J. Chromatogr. A*, vol. 1217, no. 16, 2010, pp. 2383–2389.

[118] Y. Chen, X. Gu, S. quan Huang, J. Li, X. Wang, and J. Tang, Optimization of ultrasonic/microwave assisted extraction (UMAE) of

polysaccharides from Inonotus obliquus and evaluation of its anti-tumor activities, *Int. J. Biol. Macromol.*, vol. 46, no. 4, 2010, pp. 429–435.

[119] M. Li, M. O. Ngadi, and Y. Ma, Optimisation of pulsed ultrasonic and microwave-assisted extraction for curcuminoids by response surface methodology and kinetic study, *Food Chem.*, vol. 165, 2014, pp. 29–34.

[120] Z. Lianfu and L. Zelong, Optimization and comparison of ultrasound/microwave assisted extraction (UMAE) and ultrasonic assisted extraction (UAE) of lycopene from tomatoes, *Ultrason. Sonochem.*, vol. 15, no. 5, 2008, pp. 731–737.

[121] Q. You, X. Yin, S. Zhang, and Z. Jiang, Extraction, purification, and antioxidant activities of polysaccharides from *Tricholoma mongolicum Imai*, *Carbohydr. Polym.*, vol. 99, 2014,.pp. 1–10.

[122] A. L. B. Dias, C. S. Arroio Sergio, P. Santos, G. F. Barbero, C. A. Rezende, and J. Martínez, Effect of ultrasound on the supercritical $CO_2$ extraction of bioactive compounds from *dedo de moça* pepper (*Capsicum baccatum* L. var. pendulum), *Ultrason. Sonochem.*, vol. 31, 2016, pp. 284–294.

[123] P. Santos, A. C. Aguiar, G. F. Barbero, C. A. Rezende, and J. Martínez, Supercritical carbon dioxide extraction of capsaicinoids from malagueta pepper (*Capsicum frutescens* L.) assisted by ultrasound, *Ultrason. Sonochem.*, vol. 22, 2015, pp. 78–88.

[124] B. Klejdus, L. Lojkova, M. Plaza, M. Šnóblová, and D. Štěrbová, Hyphenated technique for the extraction and determination of isoflavones in algae: Ultrasound-assisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry, *J. Chromatogr. A*, vol. 1217, no. 51, 2010, pp. 7956–7965.

[125] W. Q. Tang, D. C. Li, Y. X. Lv, and J. G. Jiang, Extraction and removal of caffeine from green tea by ultrasonic-enhanced supercritical fluid, *J. Food Sci.*, vol. 75, no. 4, 2010, pp.C363–C368.

[126] Y. C. Yang, M. C. Wei, and S. J. Hong, Ultrasound-assisted extraction and quantitation of oils from *Syzygium aromaticum* flower bud (clove) with supercritical carbon dioxide, *J. Chromatogr. A*, vol. 1323, 2014, pp. 18–27.

[127] Y. C. Yang, M. C. Wei, S. J. Hong, T. C. Huang, and S. Z. Lee, Development/optimization of a green procedure with ultrasound-assisted improved supercritical carbon dioxide to produce extracts enriched in oleanolic acid and ursolic acid from *Scutellaria barbata* D. Don, *Ind. Crops Prod.*, vol. 49, 2013, pp. 542–553.

[128] Q. F. Lu, L. F. Pan, M. Chen, Y. Qiu, and B. H. Xie, Research on the microwave-assisted supercritical $CO_2$ extraction of alkaloids from *Gynura segetum (Lour.) Merr*, *Adv. Mater. Res.*, vol. 988, 2014, pp. 390–396.

[129] H. Sereshti, R. Heidari, and S. Samadi, Determination of volatile components of saffron by optimised ultrasound-assisted extraction in tandem with dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry, *Food Chem.*, vol. 143, 2014, pp. 499–505.

[130] H. Sereshti, S. Samadi, and M. Jalali-Heravi, Determination of volatile components of green, black, oolong and white tea by optimized ultrasound-assisted extraction-dispersive liquid-liquid microextraction coupled with gas chromatography, *J. Chromatogr. A*, vol. 1280, 2013, pp. 1–8.

[131] H. Sereshti, Y. Izadmanesh, and S. Samadi, Optimized ultrasonic assisted extraction-dispersive liquid-liquid microextraction coupled with gas chromatography for determination of essential oil of *Oliveria decumbens* vent, *J. Chromatogr. A*, vol. 1218, no. 29, 2011, pp. 4593–4598.

[132] M. Roosta, M. Ghaedi, A. Daneshfar, and R. Sahraei, Ultrasound assisted microextraction-nano material solid phase dispersion for extraction and determination of thymol and carvacrol in pharmaceutical samples: Experimental design methodology, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, vol. 975, 2015, pp. 34–39.

[133] M. A. Rostagno, M. D' Arrigo, J. A. Martinez, and J. A. Martinez, Combinatory and hyphenated sample preparation for the determination of bioactive compounds in foods, *TrAC-Trends Anal. Chem.*, vol. 29, no. 6, 2010, pp. 553–561.

[134] M. C. Wei, Y. C. Yang, and S. J. Hong, Determination of oleanolic and ursolic acids in *Hedyotis diffusa* using hyphenated ultrasound-assisted

supercritical carbon dioxide extraction and chromatography, *Evid. Based. Complement. Alternat. Med.*, vol. 2015, 2015, pp. 450547-450556.

[135] J. L. P. Reategui, A. P. da Fonseca Machado, G. F. Barbero, C. A. Rezende and J. Martinez, Extraction of antioxidant compounds from blackberry (*Rubus* sp.) bagasse using supercritical $CO_2$ assisted by ultrasound, *J Supercrit Fluids*, vol. 94, 2014, pp. 223-233.

[136] Y. Wen, J. Nie, Z. G. Li, X. Y. Xu, D. Wei, and M. R. Lee, The development of ultrasound-assisted extraction/dispersive liquid-liquid microextraction coupled with DSI-GC-IT/MS for analysis of essential oil from fresh flowers of *Edgeworthia chrysantha* Lindl., *Anal. Methods*, vol. 6, no. 10, 2014, pp. 3345–3352.

[137] H. Ebrahimi-Najafabadi, R. Leardi, and M. Jalali-Heravi, Experimental design in analytical chemistry -Part I: Theory, *J. AOAC Int.*, vol. 97, no. 1, 2014, pp. 3–11.

[138] B. Dejaegher, and Y. Vander Heyden, Experimental designs and their recent advances in set-up, data interpretation, and analytical applications, *J. Pharm. Biomed. Anal.*, vol. 56, no. 2, 2011, pp. 141–158.

[139] K. M. Sharif *et al.*, Experimental design of supercritical fluid extraction - A review, *J. Food Eng.*, vol. 124, 2014, pp. 105–116.

[140] H. Sun, X. Ge, Y. Lv, and A. Wang, Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed, *J. Chromatogr. A*, vol. 1237, 2012, pp. 1–23.

[141] A. G. Carr, R. Mammucari, and N. R. Foster, A review of subcritical water as a solvent and its utilisation for the processing of hydrophobic organic compounds, *Chem. Eng. J.*, vol. 172, no. 1, 2011, pp. 1–17.

[142] S. Dey and V. K. Rathod, Ultrasound assisted extraction of β-carotene from *Spirulina platensis*, *Ultrason. Sonochem.*, vol. 20, no. 1, 2013, pp. 271–276.

[143] D. Ruen-ngam, A. Shotipruk, and P. Pavasant, “Comparison of extraction methods for recovery of astaxanthin from *Haematococcus pluvialis*,” *Sep. Sci. Technol.*, vol. 46, no. 1, pp. 64–70, 2011.

[144] X. Wang, Y. Wu, G. Chen, W. Yue, Q. Liang, and Q. Wu, Optimisation of ultrasound assisted extraction of phenolic compounds from *Sparganii rhizoma* with response surface methodology, *Ultrason. Sonochem.*, vol. 20, no. 3, 2013, pp. 846–854.

[145] Y. Xu and S. Pan, Effects of various factors of ultrasonic treatment on the extraction yield of all-*trans*-lycopene from red grapefruit (*Citrus paradise* Macf.), *Ultrason. Sonochem.*, vol. 20, no. 4, 2013, pp. 1026–1032.

[146] T. Tsiaka, P. Zoumpoulakis, V. J. Sinanoglou, C. Makris, G. A. Heropoulos, and A. C. Calokerinos, Response surface methodology toward the optimization of high-energy carotenoid extraction from *Aristeus antennatus* shrimp, *Anal. Chim. Acta*, vol. 877, 2015, pp. 100–110.

[147] F. Chemat and G. Cravotto, *Microwave-assisted Extraction for Bioactive Compounds*, Springer, 2013.

[148] M. L. Clodoveo, V. Durante, and D. La Notte, Working towards the development of innovative ultrasound equipment for the extraction of virgin olive oil, *Ultrason. Sonochem.*, vol. 20, no. 5, 2013, pp. 1261–1270.

[149] E. Ibañez, M. Herrero, J. A. Mendiola, and M. Castro-Puyana, Extraction and characterization of bioactive compounds with health benefits from marine resources: macro and micro algae, cyanobacteria, and invertebrate, *Marine Bioactive Compounds*, M. Hayes, ed, Springer, 2012, pp. 55–98.

[150] I. F. Strati and V. Oreopoulou, Recovery of carotenoids from tomato processing by-products-A review, *Food Res. Int.*, vol. 65, 2014, pp. 311–321.

[151] F. A. Reyes, J. A. Mendiola, S. Suárez-Alvarez, E. Ibañez, and J. M. Del Valle, Adsorbent-assisted supercritical $CO_2$ extraction of carotenoids from *Neochloris oleoabundans* paste, *J. Supercrit. Fluids*, vol. 112, 2016, pp. 7–13.

[152] F. A. Reyes, C. S. Sielfeld, and J. M. del Valle, Effect of high-pressure compaction on supercritical $CO_2$ extraction of astaxanthin from *Haematococcus pluvialis*, *J. Food Eng.*, vol. 189, 2016, pp. 123–134.

[153] N. Mezzomo, J. Martínez, M. Maraschin, and S. R. S. Ferreira, Pink shrimp (*P. brasiliensis and P. paulensis*) residue: Supercritical fluid extraction of carotenoid fraction, *J. Supercrit. Fluids*, vol. 74, 2013, pp. 22–33.

[154] M. Durante, M. S. Lenucci, and G. Mita, Supercritical carbon dioxide extraction of carotenoids from pumpkin (*Cucurbita* spp.): A review, *Int. J. Mol. Sci.*, vol. 15, no. 4, 2014, pp. 6725–6740.

[155] P. Sookwong, P. Suttiarporn, P. Boontakham, P. Seekhow, S. Wangtueai, and S. Mahatheeranont, Simultaneous quantification of vitamin E, γ-oryzanols and xanthophylls from rice bran essences extracted by supercritical $CO_2$, *Food Chem.*, vol. 211, 2016, pp. 140–147.

[156] J. S. Kim, C. G. An, J. S. Park, Y. P. Lim, and S. Kim, Carotenoid profiling from 27 types of paprika (*Capsicum annuum* L.) with different colors, shapes, and cultivation methods, *Food Chem.*, vol. 201, 2016, pp. 64–71.

[157] K. Zaghdoudi *et al.*, Accelerated solvent extraction of carotenoids from: Tunisian Kaki (*Diospyros kaki* L.), peach (*Prunus persica* L.) and apricot (*Prunus armeniaca* L.), *Food Chem.*, vol. 184, 2015, pp. 131–139.

[158] J. Y. Heo, S. Kim, J. H. Kang, and B. Moon, Determination of lutein from green tea and green tea by-products using accelerated solvent extraction and UPLC, *J. Food Sci.*, vol. 79, no. 5, 2014, pp. 816–821.

[159] E. Luengo, S. Condon-Abanto, S. Condon, I. Alvarez, and J. Raso, Improving the extraction of carotenoids from tomato waste by application of ultrasound under pressure, *Sep. Purif. Technol.*, vol. 136, 2014, pp. 130–136.

[160] A. J. Purohit and P. R. Gogate, Ultrasound-assisted extraction of β-carotene from waste carrot residue: Effect of operating parameters and type of ultrasonic irradiation," *Sep. Sci. Technol.*, vol. 50, no. 10, 2015, pp. 1507–1517.

[161] A. Sobolev, S. Carradori, D. Capitani, S. Vista, A. Trella, F. Marini, and L. Mannina, Saffron samples of different origin: an NMR study of microwave-assisted extracts. *Foods*, vol. 3, 2014, pp. 403–419.

[162] B. Hiranvarachat and S. Devahastin, Enhancement of microwave-assisted extraction via intermittent radiation: Extraction of carotenoids from carrot peels, *J. Food Eng.*, vol. 126, 2014, pp. 17–26.

[163] B. Hiranvarachat, S. Devahastin, and N. Chiewchan, In vitro bioaccessibility of β-carotene in dried carrots pretreated by different methods, *Int. J. Food Sci. Technol.*, vol. 47, no. 3, 2012, pp. 535–541.

[164] K. K. H. Y. Ho, M. G. Ferruzzi, A. M. Liceaga, and M. F. San Martín-González, Microwave-assisted extraction of lycopene in tomato peels: Effect of extraction conditions on all-trans and cis-isomer yields, *LWT-Food Sci. Technol.*, vol. 62, no. 1, 2015, pp. 160–168.

[165] S. Roohinejad, *Extraction of β-carotene from carrot pomace using microemulsions and pulsed electric fields*, Doctoral dissertation, Dept. of Food Science, University of Otago, 2014.

[166] E. Luengo, S. Condón-Abanto, I. Álvarez, and J. Raso, effect of pulsed electric field treatments on permeabilization and extraction of pigments from *Chlorella vulgaris*," *J. Membr. Biol.*, vol. 247, no. 12, 2014, pp. 1269–1277.

[167] E. Luengo, J. M. Martínez, A. Bordetas, I. Álvarez, and J. Raso, Influence of the treatment medium temperature on lutein extraction assisted by pulsed electric fields from Chlorella vulgaris, *Innov. Food Sci. Emerg. Technol.*, vol. 29, 2015, pp. 15–22.

[168] J. L. Markley *et al.*, The future of NMR-based metabolomics, *Curr. Opin. Biotechnol.*, vol. 43, 2017, pp. 34–40.

[169] G. A. Nagana Gowda and D. Raftery, Can NMR solve some significant challenges in metabolomics? *J. Magn. Reson.*, vol. 260, 2015, pp. 144–160.

[170] S. Bijttebier, A. Van der Auwera, K. Foubert, S. Voorspoels, L. Pieters, and S. Apers, Bridging the gap between comprehensive extraction protocols in plant metabolomics studies and method validation, *Anal. Chim. Acta*, vol. 935, 2016, pp. 136–150.

[171] C. Fotakis *et al.*, Metabolic and antioxidant profiles of herbal infusions and decoctions, *Food Chem.*, vol. 211, 2016, pp. 963–971.

[172] B. de Falco and V. Lanzotti, NMR spectroscopy and mass spectrometry in metabolomics analysis of *Salvia*, *Phytochem. Rev.*, vol. 17, no. 5, 2018, pp. 951–972.

[173] E. A. Mahrous and M. A. Farag, Two dimensional NMR spectroscopic approaches for exploring plant metabolome: A review, *J. Adv. Res.*, vol. 6, no. 1, 2015, pp. 3–15.

[174] M. D. Luque de Castro and M. M. Delgado-Povedano, Ultrasound: A subexploited tool for sample preparation in metabolomics, *Anal. Chim. Acta*, vol. 806, 2014, pp. 74–84.

[175] M. Goliomytis, H. Orfanou, E. Petrou, M. A. Charismiadou, P. E. Simitzis, and S. G. Deligeorgis, Effect of hesperidin dietary supplementation on hen performance, egg quality and yolk oxidative stability, *Br. Poult. Sci.*, vol. 55, no. 1, 2014, pp. 98–104.

[176] M. Y. Mushtaq, Y. H. Choi, R. Verpoorte, and E. G. Wilson, Extraction for Metabolomics: Access to The Metabolome, *Phytochem. Anal.*, vol. 25, no. 4, 2014, pp. 291–306.

[177] Σπυρίδων Κουτσούκος, Μελέτη διεργασίας εκχύλισης πρόπολης με Βαθέως Ευτηκτικούς Διαλύτες και χρήση του εκχυλίσματος για τη σύνθεση υβριδικών νανοσύνθετων Ag/χιτοζάνης/πρόπολης, Διπλωματική Εργασία, Σχολή Χημικών Μηχανικών, Εθνικό Μετσόβειο Πολυτεχνείο, 2018.

[178] P. Du *et al.*, Determination of astaxanthin in feeds using high performance liquid chromatography and an efficient extraction method, *J. Liq. Chromatogr. Relat. Technol.*, vol. 39, no. 1, 2016, pp. 35–43.

[179] V. J. Sinanoglou *et al.*, Factors affecting human colostrum fatty acid profile: A case study, *PLoS One*, vol. 12, no. 4, 2017, p. e0175817.

[180] M. Alañón, M. Ivanović, A. Gómez-Caravaca, D. Arráez-Román, A. Segura-Carretero,. Choline chloride derivative-based deep eutectic liquids as novel green alternative solvents for extraction of phenolic compounds from olive leaf, *Arab. J. Chem.*, 2018, in press.

[181] W. Liu, K. Zhang, J. Chen, and J. Yu, Ascorbic acid and choline chloride: A new natural deep eutectic solvent for extracting tert-butylhydroquinone antioxidant, *J. Mol. Liq.*, vol. 260, 2018, pp. 173–179.

[182] T. Tsiaka *et al.*, 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, *J. Chromatogr. B*, vol. 1096, 2018, pp. 160–171.

[183] A. Sila *et al.*, Astaxanthin from shrimp by-products ameliorates nephropathy in diabetic rats, *Eur. J. Nutr.*, vol. 54, no. 2, 2015, pp. 301–307.

[184] S. L. C. Ferreira *et al.*, Multivariate optimization techniques in food analysis–A review, *Food Chem.*, vol. 273, 2017, pp. 3-8.

[185] T. Cajka and O. Fiehn, Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry, *Trends Analyt. Chem.*, vol. 61, 2014, pp. 192–206.

[186] A. Filntisi, C. Fotakis, P. Asvestas, G. K. Matsopoulos, P. Zoumpoulakis, and D. Cavouras, Automated metabolite identification from biological fluid 1H NMR spectra, *Metabolomics*, vol. 13, no. 146, 2017, pp. 1-7.

[187] Umetrics, Sweden, https://umetrics.com/, Last visit: 17 April 2019.

[188] F. C. Marincola *et al.*, 1H NMR-based urine metabolic profile of IUGR, LGA, and AGA newborns in the first week of life, *Clin. Chim. Acta*, vol. 451, no. Pt A, 2015, pp. 28–34.

[189] R. K. Saini and Y. S. Keum, Carotenoid extraction methods: A review of recent developments, *Food Chem.*, vol. 240, no. June 2017, 2018, pp. 90–103.

[190] T. Belwal *et al.*, A critical analysis of extraction techniques used for botanicals: Trends, priorities, industrial uses and optimization strategies, *TrAC-Trends Anal. Chem.*, vol. 100, 2018, pp. 82-102.

[191] X. Xiao, W. Song, J. Wang, and G. Li, Microwave-assisted extraction performed in low temperature and *in vacuo* for the extraction of labile compounds in food samples, *Anal. Chim. Acta*, vol. 712, 2012, pp. 85–93.

[192] X. Yue, Z. Xu, W. Prinyawiwatkul, and J. M. King, Improving extraction of lutein from egg yolk using an ultrasound-assisted solvent method, *J. Food Sci.*, vol. 71, no. 4, 2006, pp. 239–241.

[193] S. Takeungwongtrakul, S. Benjakul, J. Santoso, W. Trilaksani, and M. Nurilmala, Extraction and stability of carotenoid-containing lipids from hepatopancreas of pacific white shrimp (*Litopenaeus vannamei*), *J. Food Process. Preserv.*, vol. 39, no. 1, 2015, pp. 10–18.

[194] N. M. Sachindra, N. Bhaskar, and N. S. Mahendrakar, Carotenoids in different body components of Indian shrimps, *J. Sci. Food Agric.*, vol. 85, no. 1, 2005, pp. 167–172.

[195] D. V. Dandekar and V. G. Gaikar, Microwave assisted extraction of curcuminoids from *Curcuma longa*, *Sep. Sci. Technol.*, vol. 37, no. 11, 2002, pp. 2669–2690.

[196] S. ElKhori, J. R. J. Paré, J. M. R. Bélanger, and E. Pérez, The microwave-assisted process (MAPTM1): Extraction and determination of fat from cocoa powder and cocoa nibs, *J. Food Eng.*, vol. 79, no. 3, 2007, pp. 1110–1114.

[197] B. Hiranvarachat, S. Devahastin, N. Chiewchan, and G. S. Vijaya Raghavan, Structural modification by different pretreatment methods to enhance microwave-assisted extraction of β-carotene from carrots, *J. Food Eng.*, vol. 115, no. 2, 2013, pp. 190–197.

[198] V. Pasquet *et al.*, Study on the microalgal pigments extraction process: Performance of microwave assisted extraction, *Process Biochem.*, vol. 46, no. 1, 2011, pp. 59–67.

[199] M. M. Poojary *et al.*, Innovative alternative technologies to extract carotenoids from microalgae and seaweeds, *Mar. Drugs*, vol. 14, no. 11, 2016, pp. 214-248.

[200] Y. Tian *et al.*, Ultrasonic-assisted extraction and antioxidant activity of polysaccharides recovered from white button mushroom (*Agaricus bisporus*), *Carbohydr. Polym.*, vol. 88, no. 2, 2012, pp. 522–529.

[201] G. Mercado-Mercado, E. Montalvo-González, G. A. González-Aguilar, E. Alvarez-Parrilla, and S. G. Sáyago-Ayerdi, Ultrasound-assisted extraction of carotenoids from mango (*Mangifera indica* L. 'Ataulfo') by-products on in vitro bioaccessibility, *Food Biosci.*, vol. 21, 2018, pp. 125–131.

[202] H. V. Chuyen, M. H. Nguyen, P. D. Roach, J. B. Golding, and S. E. Parks, Microwave-assisted extraction and ultrasound-assisted extraction for recovering carotenoids from Gac peel and their effects on antioxidant capacity of the extracts, *Food Sci. Nutr.*, vol. 6, no. 1, 2018, pp. 189–196.

[203] J. Song, Q. Yang, W. Huang, Y. Xiao, D. Li, and C. Liu, Optimization of trans lutein from pumpkin (*Cucurbita moschata*) peel by ultrasound-assisted extraction, *Food Bioprod. Process.*, vol. 107, 2018, pp. 104–112.

[204] B. Nayak *et al.*, Comparison of microwave, ultrasound and accelerated-assisted solvent extraction for recovery of polyphenols from *Citrus sinensis* peels, *Food Chem.*, vol. 187, 2015, pp. 507–516.

[205] K. Thirugnanasambandham, V. Sivakumar, and J. P. Maran, Microwave-assisted extraction of polysaccharides from mulberry leaves, *Int. J. Biol. Macromol.*, vol. 72, 2015, pp. 1–5.

[206] J. Prakash Maran, V. Sivakumar, K. Thirugnanasambandham, and R. Sridhar, Microwave assisted extraction of pectin from waste *Citrullus lanatus* fruit rinds, *Carbohydr. Polym.*, vol. 101, 2014, pp. 786–791.

[207] P. Christen and B. Kaufmann, New trends in the extraction of natural products: Microwave-assisted extraction and pressurized liquid extraction, *Encycl. Anal. Chem.*, 2014, pp. 1–27.

[208] A. P. Abbott, D. Boothby, G. Capper, D.L. Davies, and R. K. Rasheed, Deep eutectic solvents formed between choline chloride and carboxylic acids: versatile alternatives to ionic liquids, *J. Am. Chem. Soc.*, vol 126, no. 29, 2004, pp. 9142-9147.

[209] Y. Dai, R. Verpoorte, and Y. H. Choi, Natural deep eutectic solvents providing enhanced stability of natural colorants from safflower (*Carthamus tinctorius*). Food Chem., vol. 159, 2014, pp. 116-121.

[210] Y. R. Lee and K. H. Row, Comparison of ionic liquids and deep eutectic solvents as additives for the ultrasonic extraction of astaxanthin from marine plants, *J. Ind. Eng. Chem.*, vol. 39, 2016, pp. 87–92.

[211] E. Su, M. Yang, J. Cao, C. Lu, J. Wang, and F. Cao, Deep eutectic solvents as green media for efficient extraction of terpene trilactones from *Ginkgo biloba* leaves, *J. Liq. Chromatogr. Relat. Technol.*, vol. 40, no. 8, 2017, pp. 385–391.

[212] T. Sun, Z. Xu, and J. S. Godber, Ultrasound assisted extraction in quantifying lutein from chicken liver using high-performance liquid chromatography, *J. Chromatogr. B*, vol. 830, no. 1, 2006, pp. 158–160.

[213] B. S. Inbaraj, H. Lu, C. F. Hung, W. B. Wu, C. L. Lin, B. H. Chen, Determination ofcarotenoids and their esters in fruits of *Lycium barbarum Linnaeus* by HPLC–DAD–APCI–MS, *J. Pharm. Biomed. Anal.*, vol. 47, no. 4–5, 2008, pp. 812–818.

[214] R. C. Chiste, and A. Z. Mercadante, Identification and quantification, by HPLC-DAD-MS/MS, of carotenoids and phenolic compounds from the Amazonian fruit *Caryocar villosum*, J. Agric. Food Chem., vol. 60, no. 23, 2012, pp. 5884–5892.

[215] S. K. A. Bijttebier, E. D'Hondt, N. Hermans, S. Apers, and S. Voorspoels, Unravelling ionization and fragmentation pathways of carotenoids using orbitrap technology: A first step towards identification of unknowns, *J. Mass Spectrom.*, vol. 48, no. 6, 2013, pp. 740–754.

[216] S. M. Rivera, P. Christou and R. Canela-Garayoa, Identification of carotenoids using mass spectrometry, *Mass Spectrom. Rev.*, vol. 20, no. 3, 2013, pp. 133–138.

[217] R. B. Van Breemen, L. Dong, and N. D. Pajkovic, Atmospheric pressure chemical ionization tandem mass spectrometry of carotenoids, *Int. J. Mass Spectrom.*, vol. 312, 2012, pp. 163–172.

[218] R. MacNeill, A. Farside, V. Acharya, R. Stromeyer, B. Urbanowicz, and M. Moussallie, Implication of free cholesterol in LC–MS response enhancement, *Bioanalysis*, vol. 4, no. 10, 2012, pp. 1163–1173.

[219] S. Fraselle, V. Derop, J. M. Degroodt, and J. Van Loco, Validation of a method for the detection and confirmation of nitroimidazoles and the corresponding hydroxy metabolites in pig plasma by high performance liquid chromatography-tandem mass spectrometry, *Anal. Chim. Acta*, vol. 586, no. 1–2, 2007, pp. 383–393.

[220] H. Trufelli, P. Palma, G. Famiglini, and A. Cappiello, An overview of matrix effects in liquid chromatography-mass spectrometry, *Mass Spectrom. Rev.*, vol. 30, no. 3, 2011, pp. 491–509.

[221] S. Choi, S. Kim, J. Y. Shin, M. Kim, and J. H. Kim, Development and verification for analysis of pesticides in eggs and egg products using QuEChERS and LC-MS/MS, *Food Chem.*, vol. 173, 2015, pp. 1236–1242.

[222] A. Shrivastava and V. Gupta, Methods for the determination of limit of detection and limit of quantitation of the analytical methods, *Chronicles Young Sci.*, 2011, vol. 2, no. 1, pp. 21-25.

[223] G. Jiao *et al.*, Characterization of shrimp oil from *Pandalus borealis* by high performance liquid chromatography and high resolution mass spectrometry, *Mar. Drugs*, vol. 13, no. 6, 2015, pp. 3849–3876.

[224] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), Geneva, Switzerland, https://www.ich.org/products/guidelines, Last visit: 17 April 2019.

[225] A. G. González, M. Á. Herrador, and A. G. Asuero, Intra-laboratory assessment of method accuracy (trueness and precision) by using validation standards, *Talanta*, vol. 82, no. 5, 2010, pp. 1995–1998.

[226] J. F. Song, D. J. Li, H. L. Pang, and C. Q. Liu, Effect of ultrasonic waves on the stability of all-trans lutein and its degradation kinetics, *Ultrason. Sonochem.*, vol. 27, 2015, pp. 602–608.

[227] P. Gupta, Y. Sreelakshmi, and R. Sharma, A rapid and sensitive method for determination of carotenoids in plant tissues by high performance liquid chromatography, *Plant Methods*, vol. 11, no. 1, 2015, pp. 1–12.

[228] L. Zhao, G. Zhao, F. Chen, Z. Wang, J. Wu, and X. Hu, Different effects of microwave and ultrasound on the stability of (all-E)-astaxanthin, *J. Agric. Food Chem.*, vol. 54, no. 21, 2006, pp. 8346–8351.

[229] X. Ye, *Phytochemicals in citrus: Applications in functional foods*, CRC press, 2017.

[230] L. Zhang *et al.*, Simultaneous determination of tocopherols, carotenoids and phytosterols in edible vegetable oil by ultrasound-assisted saponification, LLE and LC-MS/MS, *Food Chem.*, vol. 258, 2019, pp. 313-319.

[231] D. Abate-Pella, D. M. Freund, J. P. Slovin, A. D. Hegeman, and J. D. Cohen, An improved method for fast and selective separation of carotenoids by LC–MS, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, vol. 1067, 2017, pp. 34–37.

[232] S. Y. Leong and I. Oey, Effects of processing on anthocyanins, carotenoids and vitamin C in summer fruits and vegetables, *Food Chem.*, vol. 133, no. 4, 2012, pp. 1577–1587.

[233] N. Martins and I. C. F. R. Ferreira, Wastes and by-products: Upcoming sources of carotenoids for biotechnological purposes and health-related applications, *Trends Food Sci. Technol.*, vol. 62, 2017, pp. 33–48.

[234] A.M. Goula, M. Ververi, A. Adamopoulou, K. Kaderides, Green ultrasound-assisted extraction of carotenoids from pomegranate wastes using vegetable oils, *Ultrason. Sonochem.*, vol. 34, 2017, pp. 821–830.

[235] M. Wenzel, I. Seuss-Baum, and E. Schlich, Influences of storage time and temperature on the xanthophyll content of freeze-dried egg yolk, *Food Chem.*, vol. 124, no. 4, 2011, pp. 1343–1348.

[236] M.N. Irakli, V. F. Samanidou, and I. N. Papadoyannis, Development and validation of an HPLC method for the simultaneous determination of tocopherols, tocotrienols and carotenoids in cereals after solid-phase extraction, *J. Sep. Sci.*, vol. 34, no. 12, 2011, pp. 1375–1382.

[237] M Y. Shen, Y. Hu, K. Huang, S. Yin, B. Chen, S. Yao, Solid-phase extraction of carotenoids, J. Chromatogr. A, vol. 1216, no. 30, 2009, pp. 5763–5768.

[238] S. Hajji, I. Younes, S. Affes, S. Boufi, and M. Nasri, Optimization of the formulation of chitosan edible coatings supplemented with carotenoproteins and their use for extending strawberries postharvest life, *Food Hydrocoll.*, vol. 83, 2018, pp. 375–392.

[239] N. M. Wade, J. Gabaudan, and B. D. Glencross, A review of carotenoid utilisation and function in crustacean aquaculture, *Rev. Aquac.*, vol. 9, no. 2, 2017, pp. 141–156.

[240] A. P. Sánchez-Camargo, M. Â. Almeida Meireles, B. L. F. Lopes, and F. A. Cabral, Proximate composition and extraction of carotenoids and lipids from Brazilian redspotted shrimp waste (*Farfantepenaeus paulensis*), *J. Food Eng.*, vol. 102, no. 1, 2011, pp. 87–93.

[241] N. Mezzomo, B. Maestri, R. L. dos Santos, M. Maraschin, and S. R. S. Ferreira, Pink shrimp (*P. brasiliensis* and *P. paulensis*) residue: Influence of extraction method on carotenoid concentration, *Talanta*, vol. 85, no. 3, 2011, pp. 1383–1391.

[242] V. Andreou, I. F. Strati, C. Fotakis, M. Liouni, P. Zoumpoulakis, and V. J. Sinanoglou, Herbal distillates: A new era of grape marc distillates with enriched antioxidant profile, *Food Chem.*, vol. 253, 2018, pp. 171–178.

[243] J. Sochor *et al.*, Mathematical evaluation of the amino acid and polyphenol content and antioxidant activities of fruits from different apricot cultivars, *Molecules*, vol. 16, no. 9, 2011, pp. 7428–7457.

[244] C. Carrera, A. Ruiz-Rodríguez, M. Palma, and C. G. Barroso, Ultrasound-assisted extraction of amino acids from grapes, *Ultrason. Sonochem.*, vol. 22, 2015, pp. 499–505.

[245] K. X. Zhu, X. H. Sun, and H. M. Zhou, Optimization of ultrasound-assisted extraction of defatted wheat germ proteins by reverse micelles, *J. Cereal Sci.*, vol. 50, no. 2, 2009, pp. 266–271.

[246] O. P. S. Rebecca, A. N. Boyce, and C. Somasundram, Isolation and identification of myo-inositol crystals from dragon fruit (*Hylocereus polyrhizus*), *Molecules*, vol. 17, no. 4, 2012, pp. 4583–4594.

[247] R. Botta, L. Berra, D. Torello Marinoni, C. Carli, M.G. Mellano, and C. Beltramo, Evaluation of apricot selections for the North Italian environment, *Acta Hortic.*, no. 862, 2015, pp. 159–167.

[248] N. Tetik and E. Yüksel, Ultrasound-assisted extraction of d-pinitol from carob pods using Response Surface Methodology, *Ultrason. Sonochem.*, vol. 21, no. 2, 2014, pp. 860–865.

[249] L. Ruiz-Aceituno, M. J. García-Sarrió, B. Alonso-Rodriguez, L. Ramos, and M. L. Sanz, Extraction of bioactive carbohydrates from artichoke (*Cynara scolymus* L.) external bracts using microwave assisted extraction and pressurized liquid extraction, *Food Chem.*, vol. 196, 2016, pp. 1156–1162.

[250] S. P. Murphy *et al.*, Dietary choline and betaine intakes vary in an adult multiethnic population, *J. Nutr.*, vol. 143, no. 6, 2013, pp. 894–899.

[251] A. Sharaf, F.A. Ahmed, and S.S. El-Saadany, Biochemical changes in some fruits at different ripening stages, *Food Chem.*, vol. 31, 1989, pp. 19-28.

[252] B. Hernández-Santos *et al.*, Effect of oil extraction assisted by ultrasound on the physicochemical properties and fatty acid profile of pumpkin seed oil (*Cucurbita pepo*), *Ultrason. Sonochem.*, vol. 31, 2016, pp. 429–436.

[253] H.R. Kim, I.D. Kim, S.K. Dhungana, M.I. Kim, and D.H. Shin, Comparative assessment of physicochemical properties of unripe peach (*Prunus persica*) and Japanese apricot (*Prunus mume*), *Asian Pac. J. Trop. Med.*, vol. 4, no. 2, 2014, pp. 97-103.

[254] D. A. Esquivel-Hernández *et al.*, Advancement of green process through microwave-assisted extraction of bioactive metabolites from *Arthrospira Platensis* and bioactivity evaluation, *Bioresour. Technol.*, vol. 224, 2017, pp. 618–629.

[255] H. Bae, S. K. Yun, J. H. Jun, I. K. Yoon, E. Y. Nam, and J. H. Kwon, Assessment of organic acid and sugar composition in apricot, plumcot, plum, and peach during fruit development, *J. Appl. Bot. Food Qual.*, vol. 87, 2014, pp. 24–29.

[256] M. Lahaye, X. Falourd, B. Quemener, M. F. Devaux, and J. M. Audergon, Histological and cell wall polysaccharide chemical variability among apricot varieties, *LWT - Food Sci. Technol.*, vol. 58, no. 2, 2014, pp. 486–496.

[257] B. Ren, C. Chen, C. Li, X. Fu, L. You, and R. H. Liu, Optimization of microwave-assisted extraction of *Sargassum thunbergii* polysaccharides and

its antioxidant and hypoglycemic activities, *Carbohydr. Polym.*, vol. 173, 2017, pp. 192–201.

[258] M. Li, X. Wen, Y. Ni, K. Wang, Z. He, and X. Chen, Optimization of ultrasound-assisted extraction of okra (*Abelmoschus esculentus* (L.) Moench) polysaccharides based on response surface methodology and antioxidant activity, *Int. J. Biol. Macromol.*, vol. 114, 2018, pp. 1056–1063.

[259] C. G. Pereira and M. A. A. Meireles, Supercritical fluid extraction of bioactive compounds: Fundamentals, applications and economic perspectives, *Food Bioprocess Technol.*, vol. 3, no. 3, 2010, pp. 340–372.

[260] D. T. Santos, P. C. Veggi, and M. A. A. Meireles, Extraction of antioxidant compounds from Jabuticaba (*Myrciaria cauliflora*) skins: Yield, composition and economical evaluation, *J. Food Eng.*, vol. 101, no. 1, 2010, pp. 23–31.

[261] A. M. Farías-Campomanes, M. A. Rostagno, M. Angela, and A. Meireles, Production of polyphenol extracts from grape bagasse using supercritical fluids: yield, extract composition and economic evaluation, J. Supercrit. Fluids, vol. 77, 2013, pp. 70–78.

[262] B. A. Souza Machado, C. Gambini Pereira, S. Baptista Nunes, F. Ferreira Padilha, and M. A. Umsza-Guez, Supercritical fluid extraction using $CO_2$: main applications and future perspectives, *Sep. Sci. Technol.*, vol 48, no. 18, 2013, pp. 2741–2760.