Review

Carotenoids in Bacteria: Biosynthesis, Extraction, Characterization and Applications

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Abstract: Natural carotenoids are secondary metabolites that exhibit antioxidant, anti-inflammatory and anti-cancer properties. These types of compounds are in high demand by pharmaceutical, cosmetic, textile and food industries, leading to the search for new natural sources of carotenoids. In recent years, the production of carotenoids from bacteria has become of great interest for industrial applications. In addition to carotenoids with C40-skeletons, some bacteria have the ability to synthesize characteristic carotenoids with C30-skeletons. In this regard, a great variety of methodologies for the extraction and identification of bacterial carotenoids has been reported and this is the first review that condenses much of this information. To understand the diversity of these carotenoids, we present their biosynthetic origin in order to focus on the methodologies employed in their extraction and characterization. Special emphasis has been made on high-performance liquid chromatography-mass spectrometry (HPLC-MS) for the analysis and identification of bacterial carotenoids. We end up this review showing their potential commercial use of bacterial carotenoids. This review is proposed as a guide for the identification of these metabolites, which are frequently reported in new bacteria strains. **Keywords:** Bacterial carotenoids; Cell disruption; HPLC separation; MS analysis; Food and textile applications

1. Introduction

Naturally occurring carotenoids are produced by eukaryotic cells such as plants, algae, fungi, and prokaryotic cells like bacteria. In addition, some animals such as flamingos accumulate carotenoids in their plumage from their diet (mollusks and crustaceans). These compounds are characterized by a striking range of colorations from red to yellow, through a spectrum of orange tones. Their characteristic colours are caused by, at least, 6 conjugated double bonds present in their polyene chain. [1,2]. The human eye is able to recognize colours within the range of 360 - 750 nm (yellow - blue). The ophthalmic colour discrimination threshold approaches approximately 2 nm, and the Vis-detector limit is at best 0.1 nm [3–5]. These natural pigments are derived from isoprenoids and are classified into two main groups: carotenes (hydrocarbon carotenoids) and oxygenated carotenoids (also known as xanthophylls). Carotenes are made of carbon and hydrogen atoms exclusively (e.g. phytoene, lycopene, β-carotene). Xanthophyll possess oxygen functional groups, including carotenols (e.g., zeaxanthin), carotenals (e.g., β-apo-8'-carotenal), carotenones (e.g., cantaxanthin), and carotenoid acids (e.g., 4,4'-Diaponeurosporenoic acid) [6–8]. To date, more than 1200 carotenoids and carotenoid precursors from 722 organisms have been reported in the Carotenoid Database (http://carotenoiddb.jp/index.html), where 324 are from bacterial sources, 251 carotenoids being exclusive to these microorganisms [9]. There are variations in the numbers and

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types of carbon units, for example, C30 [10–12], C40 [13], C45 [14], and C50 [15–18]. Chains shorter than C30 occur in some bacteria via an alternate pathway. In general, thirty-seven molecules containing C30 carotenoids have been reported [9]. The double bonds in these polyenes allows for the incorporation of environmentally free radicals, and delocalization of charges (positive or negative) along the chains [19]. The antioxidant activities of carotenoids cause them to be very sensitive to light, heat, oxygen, acidic conditions and/or basic conditions.

These compounds exhibit a broad variety of applications. Carotenoids have been reported to have benefits in treating ocular diseases due to their antioxidant, anti-inflammatory or anticancer properties [19–22]. Reviews of carotenoid applications in nutraceutical products have demonstrated the potential of these chemicals, involving carotenes such as phytoene and phytofluene and xanthophylls, such as astaxanthin and lutein [22,23]. Natural carotenoids such as astaxanthin (approved by the European Commission as food dye, E161j) and lycopene (E160d) are used as food colorants. In addition, astaxanthin is used as a dietary supplement, formulated as oils and tablets. Thus, the use carotenoids obtained from microorganisms such as *Haematococcus pluvialis* (microalgae), *Xanthophyllomyces dendrorhous* (yeast) and *Blakeslea trispora* (fungi), is an alternative to synthetic carotenoids in the food industry [24–26].

The main drawbacks of these microorganisms are their long cultivation times, and their dependence on climatic conditions. For example, astaxanthin production by *H. pluvialis* requires up to 288 hours of culture and 156 hours in *X. dendrorhous*. In addition, microalgae depend on light for the production of carotenoids [27,28]. Therefore, research on bacterial carotenoids has become an area of interests due to the short culture times and better yields compared to other microorganisms [29]. Thus, astaxanthin is obtained from the bacteria *Halobacillus trueperi MXM-16* and *Exiguobacterium Sps* after 48 and 72 hours, respectively [30,31]. Although bacteria are typically associated with human pathologies, carotenoids from bacteria are just as safe as those obtained from conventional sources such as plants and chemical synthesis. Thus, the bacterial carotenoids could also be used in pharmaceuticals, textiles, cosmetics, and food [32]. Previous reviews mainly deal with carotenoids produced by yeasts, algae, and fungi over bacteria [33–37], or are maily focused on the applications of these molecules [38–43]. As far as we know, this is the first review about bacterial carotenoids that include chemical aspects such as the biosynthesis, protocols for their extraction, separation and analysis (see Figure 1).

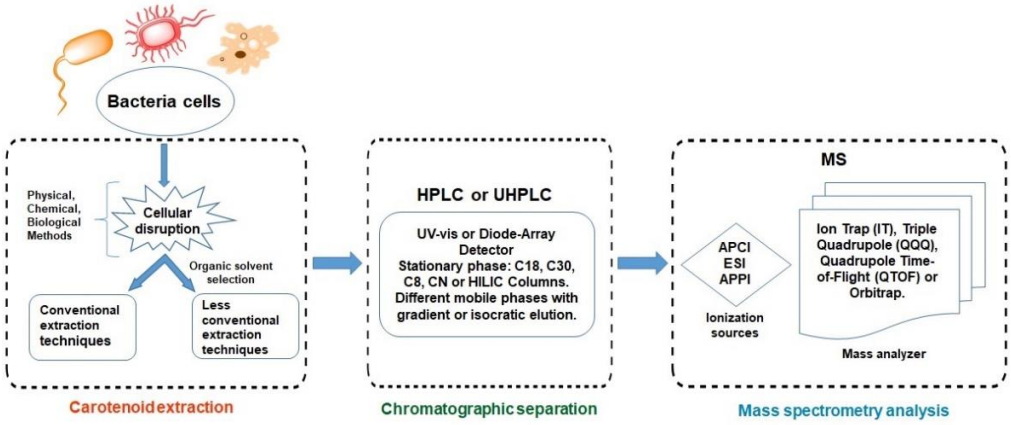

Figure 1. Schematic representation of the steps used for the characterization of bacterial carotenoids.

2. Biosynthesis of carotenoids

Carotenoids are secondary metabolites of plants and microorganisms; however, they are not involved in fundamental survival processes such as growth, development, and reproduction. They are associated with the ability to regulate the absorption of light (photooxidation), and other stress response activities [23,44,45]. Besides C40 carotenoids,

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some bacteria can synthesize C30 carotenoids and, to a lesser extent, other C45, C50, C60 chains lengths.

The biosynthesis of the carotenoids derived from bacteria starts from the C5 isoprenoid known as isopentenyl pyrophosphate (IPP), which isomerizes into dimethylallyl pyrophosphate (DMAPP) in the mevalonate (MVA) pathway (Figure 2). Initially, a condensation reaction occurs between one IPP molecule and one DMAPP molecule, producing C10-geranyl diphosphate (GPP). However, depending on the bacteria, additional units of C5 may be added from the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway [1,6,33]. Both MAV and MEP are synthesized during bacterial glycolysis. The first species initiating from acetyl CoA, and the second species derived from pyruvate. Chain elongation occurs during successive cyclical reactions, and is catalyzed by prenyl transferase (CrtE). This occurs between one or two IPP molecules with GPP, which produces C15-farnesyl pyrophosphate (FPP) and C20-geranylgeranyl diphosphate (GGPP), respectively. As a result, carotenoids derived from C30 and C40 chains take separate pathways. The synthesis of the first group occurs through the addition of two FPP units, while the second synthesis is due to two GGDP units [38,46,47]. In the C30 carotenoid group, a colourless carotenoid precursor known as 4,4'-diaphophytoene is produced, and in the C40 group the 15-cis-phytoene is the first product that is synthesized. This product is also colourless and is catalyzed by the CrtM and CrtB enzymes respectively. In both pathways, successive desaturation processes are carried out by the enzyme desaturase CrtN (C30) and CrtI (C40). In addition, Figure 2 shows the similarity in the initial steps for the biosynthetic pathways of C30 and C40 carotenoids. While the carotenoids in the C30 group are functionalized early by the oxidation processes, which produce aldehyde, ketone and/or carboxyl groups, carotenoids from the C40 group undergo dehydrogenation and cyclization processes prior to these oxidation reactions [43]. Subsequently, addition, elimination, substitution, or rearrangement reactions will produce a variety of molecules, including stereoisomers (*cis*/*trans*) and optical isomers (R/S) [47–50].

Lycopene is the first coloured carotene that is generated from four successive desaturations in the phytoene molecule. Through cyclic reactions, lycopene produces α-carotene and β-carotene, while a hydroxylation reaction in one of the β-carotene rings produces a β-cryptoxanthin molecule (Figure 2) [51]. These three pro-vitamin A carotenoids are necessary for retinol synthesis in animals. One molecule of β-carotene generates two molecules of Vitamin A, a retinoid. This vitamin is necessary for vision, cellular communication, immune function, and human reproduction [20,52,53]. Due to the poor absorption-conversion capacity of vitamin A by human body, the intake of food containing this carotenoid increases its bioavailability in the human digestive system. Xanthophylls, such as lutein, are produced by hydroxylation from α-carotene, and they have been identified in the human retina as macular pigments [54]. In addition, it has been proposed that, due to its antioxidant characteristic, lutein may be involved in filtering light that is harmful to the eye [20,21,52,55].

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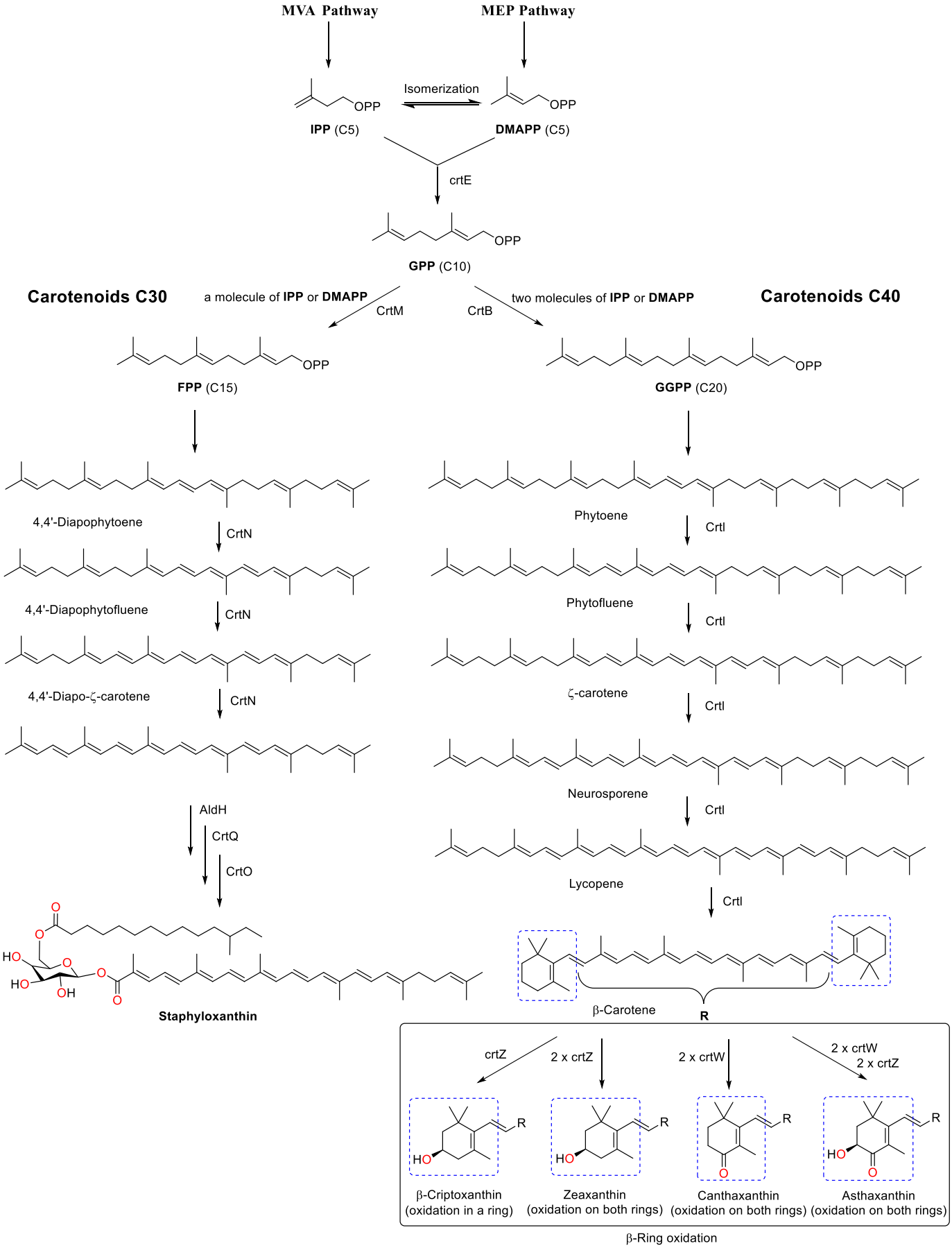

Figure 2. Scheme of bacterial carotenoids biosynthesis with C30 and C40 carbon units.

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3. Extraction Methods

There is no a single widely accepted method for extracting carotenoids from bacteria, since the yield will depend on different factors (e.g., polarity of carotenoids, moisture content, and kind of cell wall [33,56]). The extraction of carotenes is favoured by the use of non-polar solvents, such as hexane, while xanthophylls are best extracted with polar solvents, such as acetone or ethanol. In addition, the moisture of the bacterial cells will determine whether the extraction is performed by a liquid-liquid or solid-liquid process. The cell wall present in bacteria is another important factor to consider during the extraction procedure. Gram-negative bacteria have inner and outer membranes separated by a thin peptidoglycan layer and are decorated in the outer membrane by lypopolysacharide (LPS); making them generally more permeable to organic solvents. Gram-positive bacteria have an inner membrane covered by a robust cell wall composed primarily of a peptidoglycan network, making these structures more resistant to organic solvents [33]. In both cases, cell disruption of bacteria is simpler compared to microalgae and yeast, which are composed of more complex and rigid cell walls. Figure 3 shows a representation of zeaxanthin in the bacterial membrane of *Pantoea sp. YR343* [57]. Thus, cell disruption is required for the extraction of carotenoids from intact bacterial cells (Figure 3, bottom left). Zeaxanthin is also found in lipid extracts used in the preparation of vesicles (Figure 3, bottom right). As a result, the extraction of these metabolites follows a similar process, which starts with cellular disruption or cell-membrane permeabilization, followed by carotenoid solubilization with organic solvent, and further removal of unwanted compounds [2]. In addition, saponification processes are also carried out during the extraction of carotenoids, as they are often bound to sugars, proteins, and other biomolecules such as fatty acids, the latter being very common in bacterial membranes. Extractions can be performed by using conventional or modern techniques.

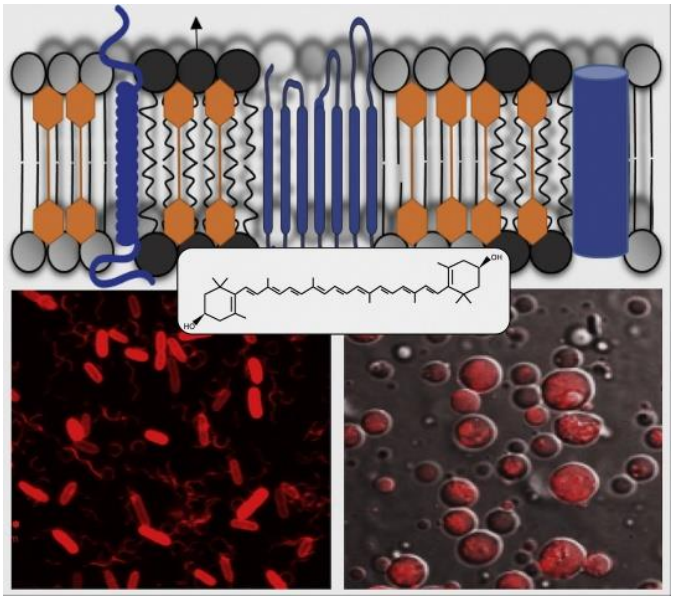

Figure 3. Representation of zeaxanthin in the bacterial membrane of *Pantoea sp. YR343* (top of the figure). Intact bacterial cells (lower left) and vesicles prepared from bacterial lipids (lower right), both observed with confocal microscopy. Reprint from Ref. [57].

3.1. Cellular disruption

Biomolecules such as proteins, sugars, and fatty acids can lead to multiple problems during the extraction of carotenoids. Thus, cell disruption is a crucial step before the extraction process. However, cell disruption involves an increase in temperature, where thermolabile compounds such as carotenoids can be affected by degradation [58]. After pelleting, cell disruption is carried out by physical, chemical, and/or biological methods

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[16]. Use of mortar and pestle, vortex mixing with or without glass beads, orbital shaking or incubation (may include temperature rise) are examples of physical methods. These approches facilitate the entry of organic solvent into bacteria, leading to solubilization of carotenoids [26,33]. Whereas chemical methods use acids, bases, or surfactants, biological methods use enzymes to cause cell disruption. Extraction techniques include conventional and non-conventional processes that may occur simultaneously during the cellular disruption. Atmospheric liquid extraction with maceration or Soxhlet extraction are typical examples of conventional techniques. Nevertheless, less conventional techniques such as microwaves (microwave-assisted extraction: MAE), ultrasounds (ultrasound-assisted extraction: UAE), supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), and gas expanded liquids (GXLs) may also be employed to simultaneously disrupt and extract carotenoids.

Comparative analysis of three cell disruption methods by means of microwaving, autoclaving, and bead milling in *Arthrospira spirulina* was conducted [59]. In microwaves, 0.5 grams of cells were used at a frequency of 2450 MHz, with 1400 W of power for 120 seconds. In the autoclaving process, 5 grams of sample were autoclaved for 30 minutes at 121 °C of temperature and 200 kPa of pressure. In the bead mill process, 150 g of *A. spirulina* cells were ground for 2 hours at 60 rpm. Water was removed from the autoclaved samples with a vacuum dryer. In this study, microwaving and bead milling resulted in the best yields for cell disruption. In a different study, cell lysis by sonication was evaluated on *Formosa sp. KMW* bacterium [60]. When an ultrasonic bath was used, yields of 139.67 ± 7.00 μg/mg biomass was obtained, while with a probe sonicator the yield was 148.9 ± 19.5 μg/mg. However, the vortex extraction method presented the highest yield of carotenoids with 1.83 ± 0.27 and 0.96 ± 0.9 mg/L respectively. Recently, Park et al. performed cell lysis on freeze-dried cells of the cyanobacterium *Arthrospira platensisis* [61]. For this extraction method, they used a pre-cooled mortar and acetone containing 0.01% butylated hydroxytoluene as antioxidant.

Another study evaluated three cell lysis methodologies in *Rhodobacter sphaeroides*. including: acidic hydrolysis with HCl, grinding, and ultrasounds-assisted extraction [62], with total carotenoids extraction yields of 4650 μg/g, 1615 μg/g and 645 μg/g, respectively. Thus, optimized conditions for HCl-assisted disruption of cell walls (30 °C, solvent-solid ratio of 30:1, for 40 min) was the best methodology. In addition, a study on the extraction of carotenoids from cyanobacteria *Synechococcus sp.* using supercritical CO_2 (SFE) was performed [63]. According to this study, the best extraction yields with SFE were achieved by applying 500 bar pressure at a temperature of 60°C. However, using 15% ethanol as extraction cosolvent in SFE, resulted in milder conditions to complete the extraction: 300 bar pressure at a temperature of 50°C. These conditions show a higher relative extraction yield compared to the conventional dimethylformamide (DMF) method. Moreover, high specificity was found in the extraction of carotenoids using SFE compared to DMF, which also extracts high amounts of chlorophylls from cyanobacteria. Comparison of cell disruption by sonication or surfactant treatment did not show a significant increase in carotenoid extraction. Therefore, the most efficient cell disruption method of *Synechococcus sp.* is achieved through the combination of pressure, temperature and ethanol [63].

Similarly, cell disruption in *Rhodothermus marinus* was achieved by pressurizing in a PLE cylinder, thus, obtaining hydroxy- and free salinixanthin [64]. Because the bacterium is Gram-negative, the authors of this study did not report a pre-treatment of this bacterium; necessary for the solvent to permeabilize the cells. However, recent results obtained in our lab, using ethyl acetate, demonstrate that macerating the cell-pellet with mortar and pestle is necessary prior to the extraction of *Staphyloccocus aureus'* (Gram-positive bacterium) carotenoids by PLE process. Regarding enzymatic degradation of the cell wall, lysozyme has been used as a biological method in the cell lysis of *Formosa sp. KMW* and *Vitellibacter sp. NMW* [65]. Initially, the cells were suspended in a saline solution followed by the addition of the enzyme to proceed with the lysis (20 mg/mL). Finally, the addition of acetone allowed the solubilization of the carotenoids. Thus, cell disruption is

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a key step that prepares the walls of bacteria for an improved performance in the subsequent or simultaneous extraction process.

3.2. *Organic solvent selection*

During or after cellular disruption, several solvents, such as hexane, acetone, dimethyl sulfoxide (DMSO), dichloromethane (DCM), ethyl acetate (AcOEt), methanol (MeOH) or ethanol (EtOH) can be used for the extraction of bacterial carotenoids, either individually or combined (see Table 1) [2,16]. The selection of the most appropriate organic solvent for each extraction will depend on the type of carotenoids present in the bacteria, as indicated above (xanthophyll or carotenes). Some bacteria only produce carotenes such as β-carotene, lycopene, etc. Therefore, the choice of the extraction solvent may be easier. However, bacteria that simultaneously produce carotenes and xanthophylls, including their esters, make the solvent selection step more complex. Therefore, a wide variety of solvent combinations has been used to obtain carotenoids from different types of samples [7], making it difficult to propose a standardized solvent or solvent mixture for a given extraction. In addition, the high oxidation sensitivity of carotenoids must be taken into account, bringing about the use of ascorbic acid, butyl-hydroxytoluene (BHT), butyl-hydroquinone (TBHQ), and butyl-hydroxyanisol (BHA), as antioxidants which stabilize carotenoids during the extraction process [2,16,66]. Very often, the analysis of bacterial carotenoids is complex because of their tendency to form ester. Therefore, the hydrolysis of the carotenoid esters before the analysis is frequently required.

3.3 *Saponification*

Bacterial carotenoid extracts contain carotenes, carotenols, carotenoid esters, carotenoid glycosides and carotenoids [7,67]. Carotenoid esters are more stable compared to carotenoids because of the increased hydrophobicity of the molecules, which prevents degradation by oxidation or isomerization due to high temperature conditions and light exposure. Furthermore, with the increase in the degree of esterification of xanthophyll, higher stability of these compounds is observed. For example, lutein is less stable than its monoester, which is less stable than the diester [67]. Despite the stability of xanthophyll esters, their identification is not easy, and a saponification process is required during sample extraction, allowing the analysis of carotenoids. Thus, saponification is an example of a chemical method used for cellular disruption and simultaneous extraction of carotenoids.

Saponification of *Bacillus spp.* cells was performed with a freeze-dried sample suspended in a solution of NaOH (10% w/v) that was sonicated for 15 to 20 minutes at room temperature [12]. Subsequently, NaOH was removed by centrifugation and the carotenoids of the saponified extract were obtained with MeOH:$CHCl_3$ (1:2) and a Tris-buffer saline. After saponification, the identification of carotenoids was easier than without saponification, showing both lycopenoate and apo-8′-lycopenoate bonded only to glucoside. In the same way, confirmation of an intermediate species in the biosynthetic pathway of *S. aureus* carotenoids was accomplished by performing a saponification of the lyophilized cells with a solution of methanolic KOH (6%) at 4 °C for 14 hours [68]. This was followed by centrifugation of 4000 rpm at 4°C and dried up by centrifugal evaporator. Afterwards, the extract was resuspended in AcOEt and NaCl solution (5.0 N) for removal of unwanted compounds. The by-product 4,4′-Diaponeurosporenoic acid was found both in *S. aureus* and *E. coli* recombinant bacteria. Since wild-type *E. coli* does not produce carotenoids, 4,4'-diaponeurosporenoic acid was obtained after insertion of genes from *S. aureus* into *E. coli* bacteria.

A comparative study of different temperatures and KOH concentrations was carried out in order to evaluate the appropriate combination for carotenoids saponification in microalgae [69]. Thus, the solvent mixture EtOH:hexane:water (77:17:6) with 0–60% KOH ((g KOH/g dry biomass)×100) at a temperatures between 25 and 80 °C were tested.

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Although the results did not allow to establish a widely applicable method for the extraction of carotenoids from the eight microalgae studied, ideal conditions were found for each strain. The researchers found that *P. reticulatum* requires a temperature of 40 °C and 10% KOH, while the best yields for *T. suecica* and *H. pluvialis* are achieved at 25 °C and 10% or 40% KOH, respectively. In *I. galbana* the ideal temperature was 60 °C and <10% KOH, while *Chlorella sp.* and *S. almeriensis* require a temperature of 80 °C and 40% KOH. Finally, for *N. gaditana* and *K. veneficum*, the best results were obtained without saponification at 60 °C. Therefore, it is not possible to establish standardized saponification conditions, since these conditions depend on the target strain, which can be extended to other microorganisms such as yeasts and bacteria.

3.4. Conventional extration techniques

Conventional techniques for carotenoids extraction have been reported in different studies. For example, *S. aureus* was incubated at 40 or 4 °C for 20 to 30 minutes, using EtOH, MeOH or acetone was used in the liquid-liquid extraction process [68,70]. Also, a secondary liquid-liquid extraction with a AcOEt:NaCl aqueous solution allow the cleaning of the extract from the very polar compounds by a salting-out effect. In both cases, several related intermediaries of the staphyloxanthin (STX) biosynthetic pathway or new carotenoid species in mutant strains were determined [68]. STX is a saccharolipid derivate from a 4,4'-diaponeurosporenoic acid, which is associated with increased membrane stiffness and resistance to antimicrobial peptide activity [71]. These studies have helped decipher the carotenoid biosynthesis in this human pathogen, allowing for the identification of a potential target for anti-virulence therapy [10]. Similarly, Rezaeeyan et al. [72] indicated that the extraction of neurosporene (a carotene with antioxidant activity and UV-B radiation protection) from *Kocuria sp. QWT-12* can be achieved by incubating with MeOH at 60° for 15 minutes.

Recently, Hartz el al. [73] studied the carotenoids 4,4′-diapophytoene, 4,4′-diapophytofluene, 4,4′-diaponeurosporene, 4,4′-diapolycopene, 4,4′-diaponeurosporenic acid from *Bacillus megaterium* using a cold extraction method with MeOH (-20°C) combined with vortex and glass beads. This method prevents thermal degradation of carotenoids, which can occur in the incubation processes. In addition, 4,4′-diaponeurosporene is a carotene which has the potential to treat inflammatory diseases. This carotene was selectively extracted from *E. coli* [74]. A similar disruption of the cells using EtOH was carried out in *Methylomonas sp. 16a* [75]. This disruption allowed the analysis of astaxanthin and canthaxanthin, that are not naturally produced by this bacterium, but obtained after genetic engineering. These carotenoids have been reported to have photoprotective, antioxidant and anti-inflammatory effects. Also, isolation and characterization of astaxanthin, adonixanthin, hydroxy-astaxanthin and dihydroxy-astaxanthin from marine Gram-negative bacteria *Brevundimonas sp.* [25] and *Erythrobacter* [13] was possible by extraction followed by shaking at 50 °C. Astaxanthin is characterized by its high antioxidant potential, which is greater than β-carotene (10 times) and vitamin E (500 times). This health benefit is often used in the field of dermatology [22]. Adonixanthin has been reported to be even stronger than astaxanthin in protecting the brain from internal haemorrhages [76].

Different expeditions have found numerous bacterial strains in Antarctica. A comprehensive study on the carotenoids produced from 30 pigmented bacterial strains isolated from Fildes Peninsula, King George Island, was reported by Vila et al. [77]. These results suggested that the antioxidant activity that carotenoids provide to these bacteria increased the resistance to extreme temperature conditions, drastic light conditions, and high doses of UV-B. Carotenoids such as zeaxanthin, β-cryptoxanthin and β-carotene were the main components obtained during extraction with MeOH. The toxicity of the selected solvent must always be considered, in particular, when scaling up the process to the industrial level. Therefore, green extraction techniques arise, which involve the use of more environmentally friendly solvents such as ethanol, ethyl acetate, or water. Due to

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the physicochemical characteristics of carotenoids, water is not a viable option in the extraction of these pigments.

3.5. *Less conventional extraction techniques*

An important aspect that may hinder the industrial application of carotenoids is the need for organic solvents during the extraction procedures, which have adverse effects on the environment and human health. Hence, the research on green chemistry approaches, which usually implies the use of less conventional extraction techniques, is of paramount importance. In addition, these techniques may offer higher yields of carotenoids in shorter periods of time when compared to traditional techniques [56]. For example, when PLE is used for carotenoids extraction, the high yield obtained is due to the higher selectivity of PLE compared to other conventional extraction techniques. As far as we know, only a few examples have been reported using PLE for the extraction of carotenoids from bacteria. Ron et al. employed PLE in the extraction of carotenoids from *R. marinus* strains DSM 4252T, DSM 4253, and PRI 493 [64], using ethanol with 1600 psi of pressure at 100 °C, during 3 extraction cycles of 2 minutes each. Four main xanthophylls were obtained: two carotenoid acyl glycoside (-hydroxyl and free) and salinixanthin (-hydroxyl and free).

Among these methods, UAE seems to be the least effective approach, even though it is the most used technique for the extraction of carotenoids from bacteria. This could be due to the low extraction temperatures used to avoid their degradation. Astaxanthin has been extracted with UAE from *Halobacillus trueperi MXM-16* bacterium using a MeOH:acetone (1:1) mixture[30]. Recently, Buddhi et al. [78] reported the spirilloxanthin series analysis of *Afifella aestuarii sp. nov.* using UAE with an acetone:MeOH (9:0.5) mixture. Also, Giuffrida et al. [18] using UAE with MeOH analyzed C50 carotenoids, such as all-E-decaprenoxanthin, all-E-sarcinaxanthin, 9-Z-decaprenoxanthin, 15-Z-decaprenoxanthin produced by strains of the cheese-ripening bacterium *Arthrobacter arilaitensis*. Extraction of zeaxanthin from *Formosa sp. KMW* and *Vitellibacter sp. NMW* was achieved by using a biological method of cell disruption which uses lysozyme with acetone in enzyme assisted extraction [65]. Similarly, cis-β-carotene, all-trans-β-carotene, chlorobactene, isorenieratene were obtained from *Rhodococcus sp. B7740* employing the same enzyme [79].

Surprisingly, despite the claimed advantages of the MAE, no reports on the use of this technique for carotenoid extraction from bacteria were found in the literature; most probably due to the time-consuming process of optimization, including solvent-sample ratio, solid-liquid ratio, and microwave power [16].

In summary, the wide variety of carotenoids produced by bacteria requires continuous development and optimization of new extraction methods. Once the carotenoids fraction is obtained, chemical characterization of this fraction is usually carried out (see Figure 1) employing HPLC-MS as described below. Table 1 presents detailed information on the methods used for the extraction of bacterial carotenoids, as well as the conditions used in their identification by HPLC-MS.

Table 1: Carotenoids obtained from different bacteria, extraction conditions and analytical methodologies applied. 1

Carotenoids	Bacteria	Filum	Gram	Extraction Technique	Characterization conditions	Ref.
All carotenoids biosynthetic pathway Staphyloxanthn.	*Staphylococcus aureus*	Firmicutes	Pos (+)	Cell without lyophilizate + EtOH + incubated water bath x 20 min at 40 °C + centrifugation + AcOEt:NaCl solution (3:1) + Na_2SO_4 (Anh.).	HPLC-UV-APCI-MS(IT): C30 column 3 µm (4.6 x 250 mm); mobile phase: (A) water, (B) acetone. Gradient elution.	[70]
Hydroxy-diaponeurosporenal, diaponeurosporenal, diapolycopenedial, diapolycopen-al-oic acid.				Cell without lyophilizate + acetone + incubate ultrasound x 30 min at 4 °C + centrifugation + AcOEt:NaCl solution (5.0 M).	HPLC-DAD-APCI-MS(IT): C18 column 2.5 µm (4.6 x 150 mm); mobile phase: (A) dwater, (B) ACN. Gradient elution.	[80]
All carotenoids biosynthetic pathway Staphyloxanthn.				Cell without lyophilizate + acetone or MeOH + centrifugation + centrifugal evaporator + resuspending in AcOEt + NaCl solution (5.0 N) + dried in centrifugal evaporator. **Saponification**: sample + incubating in MeOH at 6% KOH x 14 h at 4 °C + similar steps previously described.	HPLC-UV-vis-APCI-MS(IT): C18 column 5 µm (4.6 x 150 mm); mobile phase: ACN:MeOH:IPA (80:15:5). Isocratic elution.	[68]
Apocarotenoides family.	*Bacillus sp.*	Firmicutes	Pos (+)	Lyophilizate cell + mortar-pestle + MeOH + sonicated at r.t. x 10 - 15 min + centrifugation. Repeated extraction until lack pigment + dried in centrifugal evaporator. **Saponification**: sample + NaOH solution (10%) + sonication x 15–20 min at r. t. + centrifugation + MeOH:$CHCl_3$ (1:2) + tris-buffered saline	HPLC-DAD: C30 column 5 µm (4.6 x 250 mm) with a C30 guard column (4.6 × 20 mm); mobile phase: (A) MeOH, (B) MeOH:water (8:2) with 0.2% (w/v) ammonium acetate (C) MTBE. Gradient elution. HPLC-APCI-MS: C30 column 3 µm (2.1x150 mm) with a C30 guard column (4.6 x 20mm); mobile phase:	[12]

				+ centrifugation + collected carotenoids in chloroform phase + dried with N_2 gas.	(A) MeOH, (B) MTBE, both containing 0.1% formic acid. Gradient elution.	
4,4′-diapophytoene, 4,4′-diapophytofluene, 4,4′-diaponeurosporene, 4,4′-diapolycopene, 4,4′-diaponeurosporenic acid.	*Bacillus megaterium*	Firmicutes	Pos (+)	Cell without liofilizate + 80% MeOH (-20°C) + glass beads + vortex 30 s x 5 times + 30 s cool on ice.	HPLC-DAD-ESI-MS (QTOF): HILIC column 3.5 µm (2.1 x 100 mm); mobile phase: (A) ACN, (B) water both with 10 mM ammonium acetate (pH=6). Gradient elution.	[73]
Glycosyl-4,4′-diaponeurosporen-4′-ol-4-oic acid, 4,4′-diaponeurosporen-4′-ol-4 oic acid.	*Planococcus faecalis AJ003T*	Firmicutes	Pos (+)	Cell without lyophilizate + acetone or MeOH + centrifugation + centrifugal evaporator + AcOEt:NaCl solution (5N) + Na_2SO_4 (Anh.) + dried centrifugal evaporator.	HPLC-DAD: C18 column 2.5 µm (4.6 x 150 mm); mobile phase: (A)dwater, (B) ACN. Gradient elution. HPLC-DAD-APCI-MS: C18 column 5 µm (4.6x150 mm); mobile phase: (A) ACN:MeOH:IPA (80:15:5), (B) MeOH:water (7:3) (C) MeOH. Gradient elution.	[11,68,81,82]
Salinixanthin, carotenoid acyl glycoside	*Rhodothermus marinus*	Bacteroidetes/chlorobi group	Neg (-)	Cells washed and lyophilizate + EtOH at 100 °C with 1500 psi x 2 min in PLE (three times) + concentration.	UHPSFC-DAD-ESI-MS: (QTOF), CSH fluoro-phenyl 1.8 µm (3 x 100 mm); mobile phase: (A) CO_2, (B) MeOH with ammonium formate (0.2%). Gradient elution.	[64]

cis-synechoxanthin, all-*trans*-synechoxanthin, *cis*-β-carotene, all-*trans*-β-carotene, chlorobactene, Isorenieratene.	*Rhodococcus sp. B7740*	Actinobacteria	Pos (+)	Lyophilized cell + lysozyme + vortex x 30 s + incubation at 37°C x 1 h + centrifugation at 7000 g at 4°C x 10 min + extraction with MeOH:DCM (8:2) + centrifugation.	HPLC-DAD-APCI-MS(D-Trap-XCT) and HPLC-DAD-ESI-HRMS(LTQ-Qrbitrap): C30 column 5 µm (4.6 x 150 mm); mobile phase: (A) MeOH, (B) MTBE. Gradient elution. β-carotene, lutein and isorenieraten standards.	[79]
4-keto γ-carotene, γ-carotene.	*Rhodococcus erythropolis AN12*	Actinobacteria	Pos (+)	Cell pellets + acetone	HPLC-DAD: C8 column (4 x 125 mm); mobile phase: (A) MeOH (B) water. Gradient elution. HPLC-APCI-MS(QqQ): C18 column (4.6x250 mm); mobile phase: ACN: acetone (3:1). Isocratic elution. β-carotene, γ-carotene and lycopene standards.	[83,84]
Methyl glucosyl-3,4-dehydro-apo-8-lycopenoate.	*Planococcus maritimus MKU009*	Firmicutes	Pos (+)	Suspended cell without lyophilizate in 1.0 N NaOH + ultrasound x 5 min + centrifugation + DCM:MeOH for 3 times + contrated + suspende EtOAc:water.	Preparative silica gel HPLC (20 x 250 mm) with DCM:MeOH; preparative ODS HPLC (20 x 250 mm) with MeOH 96%, APCI- HR-MS.	[85,86]
all-*E*-sarcinaxanthin, all-*E*-decaprenoxanthin, decaprenoxanthin mono- and diglucosides, *cis*-9-*Z*-decaprenoxanthin, *cis*-15-*Z*-decaprenoxanthin,	*Arthrobacter arilaitensis*	Actinobacteria	Pos (+)	Lyophilised cells + MeOH + ultrasound x 90 min to r.t. + centrifugation + re-extracted two times with MeOH and equally MTBE.	HPLC-DAD-APCI-MS (IT-TOF): C18 column 2.7 µm (4.6 x 150 mm); mobile phase: (A) MeOH, (B) MTBE. Isocratic and gradient elution.	[18]

hydroxyspirilloxanthin.	*Roseomonas aerofrigidensis sp. nov.*	Proteobacteria	Neg (-)	Lyophilized cells + MeOH:acetone (7:2).	HPLC-DAD: C18 column, mobile phase: ACN:MeOH:AcEtOH (5:4:1). Isocratic elution.	[87,88]
Spirilloxanthin, rhodopin, anhydrorhodovibrin, tetrahydrolycopene, rhodovibrin.	*Ectothiorhodospira salini JA430T*	Proteobacteria	Neg (-)	Lyophilized cells + MeOH:acetone (7:2).	HPLC-DAD: C18 column, mobile phase: ACN:MeOH:AcEtOH (5:4:1). Isocratic elution.	[88]
trans-Diadinoxanthine, *cis*-diadinoxanthine, isomer diadinoxanthine,	*Bacillus licheniformis*	Firmicutes	Pos (+)	Cell without liofilizate + cold acetone:MeOH(7:3) + ultrasound + centrifugate.	HPLC-DAD: C18 column 5 µm (4 x 250 mm); mobile phase: ACN:MeOH. Isocratic elution.	[89]
Decaprenoxanthin and analogous.	*Arthrobacter psychrochitiniphilus 366*	Actinobacteria	Pos (+)	Lyophilized cells + MeOH + shake 5 min + concentration + hexane + shake 10 min + NaCl solution (10%) + shake + organic layer collected + re-extracted hexane:diethyl ether (3:1) and saponificate analysis.	UPLC-APCI-MS(QqQ): C18 column 1.7 µm (2.1 × 150 mm); mobile phase: (A) ACN:MeOH (7:3), (B) water. Gradient elution.	[17]
Decaprenoxanthin, mono- and di-glycosylated decaprenoxanthin.	*Arthrobacter sp. M3*	Actinobacteria	Pos (+)	Cells without lyophilize + MeOH:acetone (1:1) + dryness.	No Report	[33,90]
Saproxanthin, dehydroflexixanthin, 20-Isopentenyldehydrosaproxanthin.	*Rhodopirellula rubra LF2T*	Planctomycetes	Neg (-)	Cells without lyophilizate + solvent extracts.	No Report	[14]
Saproxanthin, dehydroflexixanthin 20-Isopentenyldehydrosaproxanthin.	*Rubinisphaera brasiliensis Gr7*	Planctomycetes	Neg (-)	Cells without lyophilizate + solvent extracts.	No Report	[14]
3,4-didehydrorhodopin, rhodopin, rhodovibrin, spirilloxanthin, anhydrorhodovibrin, lycopene.	*Rhodopseudomonas palustris CQV97*	Proteobacteria	Neg (-)	Cell without lyophilizate + acetone:MeOH (7:2) + ultrasound until colorless.	HPLC-ESI-MS(IT): C18 column (4.6 x 150 mm); mobile phase: (A) ACN:water (95:5), (B) MeOH. Isocratic elution.	[91]

Hydroxydemethylspheroidene and unkouwn carotenoid.	*Hoeflea olei sp. nov.*	Proteobacteria	Neg (-)	Lyophilized cells + MeOH:acetone (7:2) + ultrasound + centrifugate + dryness.	HPLC-DAD: C18 column (4.6 x 250 mm); mobile phase: ACN:MeOH: AcEtOH (5:4:1). Isocratic elution. HPLC-ESI-MS: C18 column (4.6 x 50 mm); mobile phase: ACN:MeOH (6:4). Isocratic elution.	[92,93]
Spirilloxanthin	*Afifella aestuarii sp. nov.*	Proteobacteria	Neg (-)	Lyophilized cells + acetone:MeOH (9:0.5) + ultrasound + centrifugation + dryness.	HPLC-DAD: C18 column (4.6 x 250 mm); mobile phase: ACN:MeOH: AcEtOH (5:4:1). Isocratic elution. HPLC-ESI-MS: C18 column (4.6 x 50 mm); mobile phase: ACN:MeOH (6:4). Isocratic elution.	[78,92]
(2′S)-deoxymyxol 1′-glucoside (2′S)-4-ketodeoxymyxol 1′-glucoside.	*Gordonia terrae AIST-1*	Actinobacteria	Pos (+)	Lyophilized cells + acetone: MeOH (7:2) + ultrasound + centrifugation + organic solvent evaporation.	HPLC-DAD: C18 column (8 x 100mm); mobile phase: (A) water (B) $CHCl_3$. Gradient elution.	[94]
Flexirubin-type	*Chryseobacterium sp. kr6*	Bacteroidetes	Neg (-)	Lyophilized cells + acetone + ultrasonic + centrifugation + dried with N_2 (gas).	HPLC-DAD-APCI-MS: C18 column (4.6 x 250 mm); mobile phase: (A) water, (B) MeOH both with formic acid (0.1%). Gradient elution	[95]
Isorenieratene, 3-hydroxyisorenieratene, 3,3′-dihydroxy-isorenieratene.	*Brevibacterium linens*	Actinobacteria	Pos (+)	Cell without lyophilize + MeOH + 50 rpm agitation x 2 hours + centrifugation + Na_2SO_4 (Anh.) + evaporation under vacuum.	HPLC-DAD: C18 column 5 µm (4.0 x 250 mm); mobile phase: (A) MeOH, (B) $CHCl_3$. Gradient elution. Lycopene, β-carotene, cryptoxanthin, astaxanthin, zeaxanthin canthaxanthin, and lutein standards.	[96]

Luteine, lycopenal, lycopenal glucoside, rhodopin, rhodopinal, rhodopinol, rhodopinglucoside.	*Rhodoblastus sphagnicola sp. Nov*	Proteobacteria	Neg (-)	Cells without lyophilize + MeOH:acetone (1:1) + ultrasonid + centrifugate + dryness.	HPLC-DAD: C18 column 5 µm (4.0 mm x 250 mm); mobile phase: (A) ACN, (b) water (C) AcOEt. Gradient elution	[97]
Canthaxanthin, phytoene, astaxanthin, lycopene, phytofluene, tetrahydrosqualene, 3-hydroxyechinenone, bisanhydrobacterioruberin, monoanhydrobacterioruberin, bacterioruberin, haloxanthin.	*Haloferax alexandrinus* GUSF-1 (KF796625)	Euryachaeota	Neg (-)	Cell-free supernatant + cold EtOH + overnight at -20°C + centrigutation.	HPLC-DAD-ESI-MS(QTOF): UHPLC and Nano-HPLC; C-18 column 3 µm (2.1 x 100 mm). Mobile phase: (A) water with 0.1% formic acid (B) ACN:MeOH (7:3).	[98]
Astaxanthin, adonixanthin, hydroxy-astaxanthin, dihydroxy-astaxanthin.	*Brevundimonas sp.*	Proteobacteria	Neg (-)	Cell without lyophilizate suspended DMSO + shake at 50°C + MeOH + centrifugation.	HPLC-DAD: C18 column 5 µm (4.6 x 35 mm); mobile phase: ACN:MeOH:IPA (85:10:5) and ACN:water (9:1). Isocratic elution. HPLC-ESI-MS: C18 column 5 µm (4.6 x 35 mm); mobile phase: (A) water, (B) MeOH. Gradient elution. β-carotene and astaxanthin standards.	[25,99–101]
2,2′-dihydroxy-astaxanthin, astaxanthin.	*Brevundimonas scallop*	Proteobacteria	Neg (-)	Lyophilized cells + acetone + rotary shaker + centrifugation + dried with N_2 (gas)	HPLC-DAD and HPLC-ESI-MS; C18 column (Hypersil ODS); mobile phase: MeOH:water (95:5). Isocratic elution.	[102]

Astaxanthin and astaxanthin isomers.	*Sphingomonas astaxanthinifaciens*	Proteobacteria	Neg (-)	Cells without lyophilize + MeOH + rotary shaker at 50°C + centrifugation	HPLC-DAD: C18 column 5 µm (4.6 × 35 mm); mobile phase: MeOH:water (9:1). Isocratic elution. HPLC-MS; Shim-Pack FC-ODS 5 µm (4.6 × 150 mm); mobile phase: MeOH:water (95:5). Isocratic elution. β-carotene, zeaxanthin standars.	[103]
Astaxanthin and 4-ketozeaxanthin	*Agrobacterium aurantiacum*	Proteobacteria	Neg (-)	Cells without lyophilize + acetone + vacuum distillation + AcOEt:water + organic phase with Na_2SO_4 (Anh.) + vacuum distillation.	HPLC-DAD: TSK gel CN 80Ts (7.8 x 300 mm); mobile phase: n-hexane:DCM:EtOH:N-ethyldiiso propylamine (80:20: O.S:O.S). Isocratic elution. EI-MS. Astaxanthin standard.	[101]
Astaxanthin	*Halobacillus trueperi MXM-16*	Firmicutes	Pos (+)	Cell without lyophilizate + MeOH:acetone (1:1) + ultrasound x 2 min + centrifugation.	HPLC-DAD: C18 column 5 µm (4x25 mm); mobile phase: MeOH. Isocratic elution.	[30]
	Exiguobacterium Sps	Firmicutes	Pos (+)	Cell pellet wash sterile water + suspended 5 mL MeOH + incubated water bath at 60 °C x 15 min + centrifugation	HPLC-UV-vis: C18 column (4.6 x 250 mm); mobile phase: (A) water, (B) MeOH. Gradient elution.	[31]
	Sphingomonas faeni ISY	Proteobacteria	Neg (-)	Cells without lyophilize + sequential extraction with hexane, $CHCl_3$ and MeOH + overnightin an orbital shaker + re-extraction + centrifugation for 15 min at 4°C + solvent evaporation under vacuum.	HPLC-UV-vis: C18 column; mobile phase: acetone:MeOH:THF (70:28:2). Isocratic elution. HPLC-MS(QqQ): C18 column; mobile phase: water (A) and ACN (B) both with 0.1% formic acid. Gradient elution.	[104]

Astaxanthin, cantaxanthin, lutein.	*Gordonia alkanivorans strain 1B*	Actinobacteria	Pos (+)	Cells dried at 55°C x 1 h + DMSO + orbital shaker at 50 °C + centrifugate + repeated until colorless + acetone:NaCl solution (20%):AcEtOH + concentration phase AcEtOH.	HPLC-DAD: C18 column (4.6 x 250 mm); mobile phase: (A) MeOH (with 0.2% water), (B) ACN. Isocratic elution	[105]
Astaxanthin and canthaxanthin.	*Methylomonas sp. 16a*	Proteobacteria	Neg (-)	Cells without lyophilize + glass beads + EtOH + vortex + DCM + vortex + centrifuge and dried.	HPLC-DAD: NP column 5 µm (4.6 x 250 mm); mobile phase: acetone:hexane (14:86). Isocratic elution.	[75,106]
Cantaxanthin	*Bradyrhizobium sp.*	Proteobacteria	Neg (-)	Cells without lyophilize + MeOH:acetone (7:2) + solution NaCl (10%) + hexane + organic phase vacuum distillation.	HPLC-DAD: Lichrosorb Si60 5 µm (4.5 x 100 mm); mobile phase: DCM:AcOEt (95:5). FAB-MS: m-nitrobenzyl alcohol–glycerol:water with TCA (1%). Isocratic elution.	[107]
Canthaxanthin isomers	*Dietzia sp.*	Actinobacteria	Pos (+)	Lyophilized cells + $CHCl_3$:MeOH (1:1) + rotavapor + resuspended in cold acetone + centrifugation + stored at -20°C with N_2 (gas).	HPLC-DAD, HPLC-APCI-MS/MS: C18 column (4.6 × 150 mm); mobile phase: ACN:MeOH (7:3); Isocratic elution. Astaxanthin, β-carotene and all-*trans*-canthaxanthin standards.	[108]
Zeaxanthin, β-cryptoxanthin, β-carotene, phytoene.	*Zobellia laminarie 465*	Bacteroidetes	Neg (-)	Lyophilized cells + MeOH + shake 5 min + hexane with 10 min shake + solution NaCl (10%) + shake + organic layer collected + re-extraction hexane:diethyl ether (3:1) and saponificate analysis.	HPLC-DAD: C18 column 1.7 µm (2.1 ×150 mm); mobile phase: (A) ACN, (B) MeOH, (C) water. Gradient elution.	[17]
Zeaxanthin, cryptoxanthin, myxoxanthophyll.	*Synechococcus sp. strain PCC 7002*	Synechococcaceae	Neg (-)	Cell without lyophilizate + acetone:MeOH (7:2) or pure MeOH + ultrasound + centrifugation.	HPLC-DAD: C18 column 5 µm (4.6 mm x 250 mm); ACN:MeOH:water (with 10 mM NH_4OAc).	[44,109]

Zeaxanthin	*Mesoflavibacter zeaxanthinifaciens*	Bacteroidetes	Neg (-)	Cells without lyophilize + MeOH + rotary shaker at 50°C + solution NaCl (10%) + hexane + organic phase vacuum distillation.	HPLC-DAD: C18 column 5 µm (4.6 mm × 35 mm); mobile phase: MeOH:water (9:1). Isocratic elution. HPLC-MS; Shim-Pack FC-ODS 5 µm (4.6 × 150 mm); mobile phase: MeOH:water (95:5). Isocratic elution. β-carotene, zeaxanthin standards.	[110]
	Formosa sp. KMW	Bacteroides	Neg (-)	Cells suspended in sterile saline + lysozyme (20 mg/ml) + acetone + Filter + hexane + Na_2SO_4 (Anh).	HPLC-DAD: C18 column (4.6 x 250 mm); mobile phase: (A)MeOH, (B)water, (C) THF. Gradient elution.	[65]
	Vitellibacter sp. NMW	Bacteroides	Neg (-)	Cells suspended in sterile saline + lysozyme (20 mg/ml) + acetone + filter + hexane + Na_2SO_4 (Anh.).	HPLC-DAD: C18 column (4.6 x 250 mm); mobile phase: (A)MeOH, (B)water, (C) THF. Gradient elution.	[65]
	Synechococcus elongatus PCC 7942	Cyanobacteria	Neg (-)	Dry cell + MeOH + centrifugation + re-suspended MeOH + sonication + centrifugation.	HPLC, LC-ESI-QTOF-MS: C18 column; mobile phase: (A) MeOH:ACN:water (21:16.5:62.5) with 10mM ammonium acetate, (B) MeOH:ACN:AcOEt (50:20:30). Gradient elution. Zeaxanthin and β-carotene standards.	[111]
	Paracoccus zeaxanthinifaciens ATCC 21588	Proteobacteria	Neg (-)	Cell pellet wash sterile water + THF + centrifugation.	HPLC-DAD: C30 column 5 µm (4.6 x 250 mm) with a C18 guard column (20 mm); mobile phase: (A) MeOH, (B) MTBE (8:2). Gradient elution. Astaxanthin, 13-*cis*-zeaxanthin, 15-*cis*-zeaxanthin, all-*trans*-zeaxanthin,	[112]

					canthaxanthin, cryptoxanthin, β-carotene, and lycopene standards.	
Lycopene, β-carotene, zeaxanthin, canthaxanthin, astaxanthin.	*Corynebacterium glutamicum*	Actinobacteria	Pos (+)	Cell resuspende + MeOH:acetone (7:3) with 0.05% BHT at 60 °C x 15 min with vortex every 5 min + centrifugation.	HPLC-DAD: C18 column 5.0 µm (4 × 125 mm) with precolumn (40 × 4 mm); mobile phase: MeOH:water (9:1). Isocratic elution and C30 column 5.0 µm (4 × 250 mm) with pre-column (4 × 10 mm); mobile phase: (A) MeOH, (B) MTBE, (C) AcEtOH. Gradient elution. Astaxanthin, β-Carotene, canthaxanthin, decaprenoxanthin, lycopene and zeaxanthin standards.	[66]
Adonixanthin	*Erythrobacter*	Proteobacteria	Neg (-)	Cell without lyophilizate + DMSO + Shake at 50 °C + MeOH + vortex + centrifugation	HPLC-DAD: C18 column 5 µm (4.6 x 35 mm); mobile phase: ACN:MeOH:THF (5.8:3.5:0.7) and ACN:water (9:1). Isocratic elution. LC-ESI-MS: Shim-Pack FC-ODS 5 µm (4.6 x 150 mm); mobile phase: (A) MeOH (B) water (95:5). Gradient elution. β-carotene, astaxanthin, zeaxanthin, canthaxanthin, and echinenone standards.	[13]
Neurosporene, α-carotene, canthaxanthin, astaxanthin, β-carotene, neoxanthin, zeaxanthin, phytofluene.	*Asaia lannensis*	Proteobacteria	Neg (-)	Cell without lyophilizate + wash PBS solution + acetone:MeOH (7:3) + shake in darkness at 60°C x 2 hours + centrifugation 6500 rpm x 10 min.	HPLC-DAD-ESI-MS(Orbitrap): C30 column 2.6 µm (3.0 x 100 mm); Mobile phase: (A) water, (B) MeOH, (C) MTBE. Gradient elution.	[113]

	Asaia bogorensis	Proteobacteria	Neg (-)	Cell without lyophilizate + wash PBS solution + acetone:MeOH (7:3) + shake in darkness at 60°C x 2 hours + centrifugation 6500 rpm x 10 min.	HPLC-DAD-ESI- MS(Orbitrap): C30 column 2.6 µm (3.0 x 100 mm); mobile phase: (A) water, (B) MeOH, (C) MTBE. Gradient elution.	[113]
Neurosporene	*Kocuria sp.QWT-12*	Actinobacteria	Pos (+)	Cells washed sterile water + MeOH + incubated water bath at 60 °C x 15 min until colorless + filter.	No Report	[72]
	Rhodobacter viridis JA737	Proteobacteria	Neg (-)	Dry cell mass + acetone:MeOH (9:0.5) + sonication + centrifugation + solvent evaporation with rotary evaporator.	HPLC-DAD; C18 column (4.6 x 250 mm); mobile phase: ACN:MeOH:AcOEt (5:4:1). Isocratic elution. HPLC-ESI-MS; C18 column (50 x 4.6 mm); mobile phase: ACN:MeOH (6:4). Isocratic elution.	[92]
β-carotene, zeaxanthin, astaxanthin, keto-myxocoxanthin glucoside-ester, diapolycopenedioic-acid-diglu- cosyl-ester.	*Exiguobacterium acetylicum S01*	Firmicutes	Pos (+)	Cells without lyophilize + mortar and pestle + MeOH:acetone (7:3) + centrifugation + organic solvent evaporation.	HPLC-DAD-APCI-MS(IT); C18 column (2.1 × 50 mm); mobile phase: (A) ACN (B) MeOH, both containing 0.5% MTBE. Gradient elution.	[39,114,115]
β-cryptoxanthin, β-carotene	*Pseudomonas sp.*	Proteobacteria	Neg (-)	Cell without lyophilize + MeOH + tris-HCl solution (50 mM, pH=7.5) + NaCl solution (1M) + $CHCl_3$ + mixed x 5 min + centrifugation + lower phase evaporation.	HPLC-DAD; TSKgel ODS-80s column 5 µm (4.6 × 150 nm); mobile phase: (A) MeOH:water (95:5) and (B) MeOH:THF (7:3). Gradient elution. β-cryptoxanthin and β-carotene standards.	[116]

The different solvents correspond to volume-volume ratios. Ahn.: anhydrous, ACN: acetonitrile, THF: tretrahydrofuran, MTBE: methyl tert-butyl ether IPA: isopropanol and HR-MS: high-resolution mass spectrometry.

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4. Characterization

The complex carotenoid extract from bacteria needs to be separated before the different compounds can be identified. Hence, thin layer chromatography (TLC) and open column chromatography (OCC) have been traditionally employed for this separation step. These techniques involve off-line carotenoid separation frequently followed by mass spectrometric identification, as will be discussed in the next section. The major drawbacks of these techniques are the high sample concentration required and their low resolution, which curtails the ability to separate the wide variety of chemical structures reported for carotenoids and their intermediates (>1200 compounds). This complexity includes isomers (*cis* or *trans*), or modifications in the main chain that occur as a result of cyclization, addition of side chains, oxidation, hydrogenation, or dehydrogenation. Many of these structures are usually present in the bacterial carotenoid extract, making both their separation and identification very challenging. In addition, not all the carotenoid standards or their degradation products are available, which causes additional limitations to their identification [2].

Currently, the technique of choice to face all these analytical challenges is high-performance liquid chromatography (HPLC), usually coupled to a diode array detector (DAD) (or UV-visible detector) and mass spectrometry (MS). Therefore, the following paragraphs will discuss the HPLC separation methods reported on bacterial carotenoid extracts and analysis by DAD and MS.

4.1. Chromatographic separation

HPLC may use different types of columns available on the market such as cyano (CN), octylsilane (C8), octadecylsilane (C18) or other more specific columns for separation of carotenoids (and some isomers) such as C30 [52]. Several reports have used C18 stationary phase columns in the analysis of bacterial carotenoids (see Table 1). The drawback of C18 columns is the poor resolution in the separation of *cis*/*trans* isomers of carotenes with 40 or more carbon atoms. However, when xanthophylls are analyzed, similar profiles are observed using C18 and C30 columns [2,117]. Features such as better stability at a high pH, higher speed and low cost make the C18 column useful and convenient for simple carotenoid analysis compared to the C30 column [118]. C18 is also useful for other types of separation (vide infra). However, the higher retention capacity of both polar and non-polar compounds in C30 columns allows for a greater separation of carotenoids, thus favouring the resolution of geometric isomers, which is highly useful in complex carotenoid mixtures. Furthermore, C30 columns are stable at high pH and are more resistant to phase collapse than C18 columns. However, C30 columns generally require much longer analysis time than C18 columns.

Recently, Turcsi et al. [119] compared the separation capacity of C18 and C30 stationary phases using a 100 carotenoids sample divided into six main groups: carotenes, hydroxycarotenoids, epoxycarotenoids, ketocarotenoids, and reduced κ carotenoids. The elution order of carotenoids in C18 columns was according to the polarity of each compound, with hydroxycarotenoids as the first eluted compounds, followed by carotenes, keto- and epoxy-carotenoids, which showed longer retention times. Similarly, the length of the molecule and the presence/number of polyenes are proportional to the retention times in C30 phases. Thus, the separation of regioisomers from compounds such as zeaxanthin and lutein, including their epoxides, is best achieved with the C30 stationary phase. In contrast, C18 columns are efficient at separating polar carotenoid stereoisomers and epimers. Therefore, when analysing an unknown extract, the initial analysis with C18 phase is recommended, and the characterization is complemented using a C30 phase [117].

Another example of the versatility of the C18 stationary phase in ultra-high-pressure liquid chromatography with a diode array detector (UHPLC-DAD) was reported for the separation and quantification of 13 carotenoids including three phytoene isomers, two-carotene isomers, and two astaxanthin isomers [120]. Analysis was performed in a BEH

C18 column and a Cortecs C18 column (2.1×50 mm) at 35 °C with a flow rate of 400 µl/min, employing MeOH and ACN as mobile phase, with relatively short analysis times of 9 and 12 minutes. The method employed for both columns demonstrated a high level of reproducibility. However, the BEH C18 (1.7 µm) showed a slightly higher performance than Cortecs C18 (1.6 µm) in terms of recovery and less precision for the analysed carotenoids. Selectivity of the chromatographic method was demonstrated by analysing a carotenoid extract from *H. pluvialis*. Thus, this method could be applied not only to algae samples, but also to bacteria. Moreover, with the goal of producing a fast and efficient chemical identification method of these bacterial metabolites, Asker's group and others have developed robust of screening and profiling methods for the simultaneous analysis of diverse strains of bacteria producing carotenoids, which has emerged as an efficient alternative [13,99]. Meanwhile, a HPLC–MS/MS method for the analysis of microbial carotenoids and quinones produced by five bacteria (*Brevibacterium linens, Micrococcus luteus, Panthoea agglomerans*, *Rhodococcus equi*, *Arthrobacter bergerei*, and *Arthrobacter protophormiae*) used a C18 column. This method allowed the structural elucidation of acyclic carotenes, mono and bicyclo carotenes, mono and bicyclo xanthophylls and acyclic xanthophylls [121].

Other reports have indicated the versatility of C8 columns in the separation of carotenoids. Thus, depending on the complexity of the carotenoid mixture, C8 columns have the advantage of shorter retention times compared to C18 and C30 columns; generally, C8 columns take around 20 minutes less than C18 and C30 columns [83,84,122]. Thus, it was possible to analyse fucoxanthinol, fucoxanthin, mactraxanthin, violaxanthin, neoxanthin and auroxanthin. It should be pointed out that the latter was an isomer of violaxanthin at acidic pH [122].

In 2017, Abate-Pella et al. [123] reported the use of a CN column in the efficient separation of more than 11 carotenoid standards in 9 or 18 minutes using MeOH as organic solvent. These cartenoids included carotene isomers (α-carotene, β-carotene and lycopene) and xanthophyll isomers (canthaxanthin, α-cryptoxanthin and β-cryptoxanthin). The applicability of this chromatographic method was demonstrated on three biological samples of strawberry leaf, chicken feed supplement, and the bacterium *Chloroflexus aurantiacus*. The resolution factor was 0.75 for the three carotenoids present in this bacterium. In addition, *cis*/*trans* isomerization of α-carotene was observed in the form of two peaks in the chromatogram for this carotene.

In summary, HPLC is the (gold) standard technique for carotenoid separation. However, it is worth noting that carotenoids are influenced by the physical properties of the solvents used. Therefore, the selection of a suitable solvent in the mobile phase can lead to drastic changes in the chromatographic separation. For this reason, ACN, MeOH, THF, $CHCl_3$, d-water, among other solvents, have been used (Table 1). In recent years, there has been an increase in the report of MeOH:MTBE:water mixtures as mobile phase with modifiers, such as ammonium acetate, ammonium formiate, acetic acid or formic acid, among others [67,117,124–127], since their performances in the separation and identification of carotenoids has worked well (Figure 4).

However, many studies do not report the quantification of all carotenoids present in a sample because of the complexity of carotenoid chromatograms due to the high variety of isomers and structurally related compounds. In many cases, the standards of the carotenoids that are being analysed are not available, or only one of the isomers is available. Carotenoid quantification requires the use of the specific standard. Alternatively, when this is not possible, a different carotenoid, typically β-carotene is employed [128]. Even if chromatographic separation is achieved, the lack of standards still makes difficult to characterize this family of compounds. Mass spectrometry emerges as a useful technique for the identification and quantification of carotenoids.

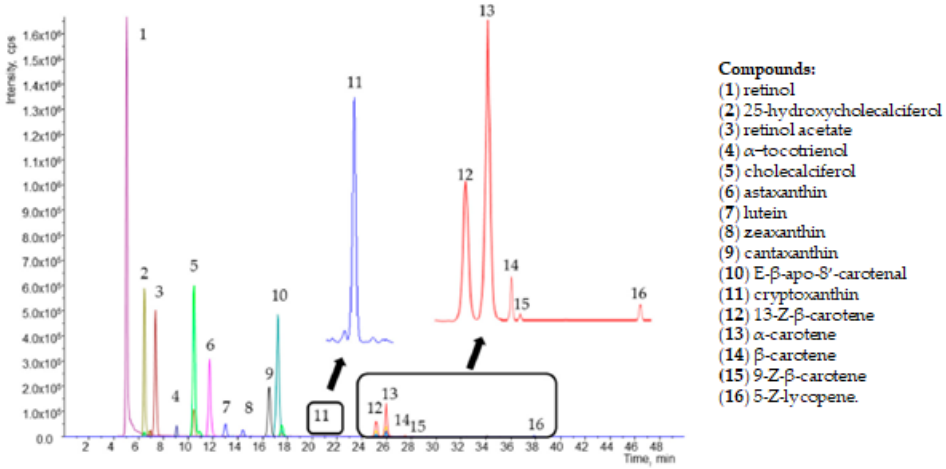

Figure 4. Chromatographic separation of mixed standards of carotenes and xanthophylls on a C30 column using the mixture MeOH:MTBE:water with ammonium acetate. Adapted from Ref. [124].

4.2. *Mass spectrometry analysis*

Mass spectrometry (MS) provides structural information of the target compound though the molecular mass and fragmentation patterns that can be obtained in tandem mass spectrometry (MS/MS) mode [2,56]. MS selectivity allows the identification of compounds that cannot be separated by HPLC due to coelution. Therefore, HPLC-MS overcomes some of the problems related to the use of HPLC-DAD or HPLC-UV-vis for the identification of carotenoids since different families of these compounds have the same absorption in the UV-visible region.

In early studies, the identification of carotenoids was carried out using ionization methods such as electron ionization (EI), negative ion chemical ionization (CI) and fast atom bombardment (FAB) [56,129–131]. The main drawback of EI and CI methods is the carotenoids thermolability. These ionization sources require solvent removal at relatively high temperatures prior to compounds ionization. Although FAB was also implemented for LC-MS analysis of carotenoids, this ionization technique presents low ionization capacity in negative mode [129,131,132]. In addition, matrix-assisted laser desorption ionization (MALDI) has also been used in the identification of carotenoids [133–135]; unfortunately, MALDI does not allow the hyphenation of HPLC and MS; thus, complex samples need to be previously fractionated off-line. For example, Yoshida et al. used TLC for the separation of carotenoids from *Bacillus subtilis* followed by MALDI-TOF MS [136]. Similarly, off-line identification of carotenoids in *Haloferax mediterranei* was achieved using TLC-MALDI-TOF MS [137]. In addition, Schöner et al. [134] reported the characterization of bacterial carotenoids using preparative HPLC separation and identification by MALDI-TOF MS.

Two main ionization sources are currently used for carotenoid analysis: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). While ESI is useful in the analysis of ionic and polar compounds such as xanthophylls [117,138], APCI allows the characterization of both, carotenes and xanthophylls [117,132]. In recent years, a new ionization source, which is suitable for non-polar compounds, has been introduced. This source is known as atmospheric pressure photoionization (APPI) [139]. The ionization of metabolites in APPI is improved with the use of dopants; however, there is no universal solvent for this source, and the technique is still under development [117,139].

In APCI it is possible to use mobile phases without buffers and with high flow rates (greater than 800 µL/min), while ESI depends on the use of buffers or organic modifiers for efficient ionization and mobile phase flow rates lower or equal to 500 µL/min. In addition, ESI, and NaOH solutions (0.5 mmol/L) have been used to increase the Na^+ adducts of carotenoids, thus, improving the sensitivity of this ionization source [64].

Another characteristic of APCI is the possibility of distinguishing some isomeric carotenoids by comparison of the fragmentation patterns between the positive and negative modes [132,139]. Thus, the best ionization source for the identification of carotenes and xanthophylls is still APCI. Examples of positive and negative ionization in APCI are presented in Figures 5 and 6, showing the different sensitivity and selectivity that these two ionization processes can provide to the carotenoids analysis.

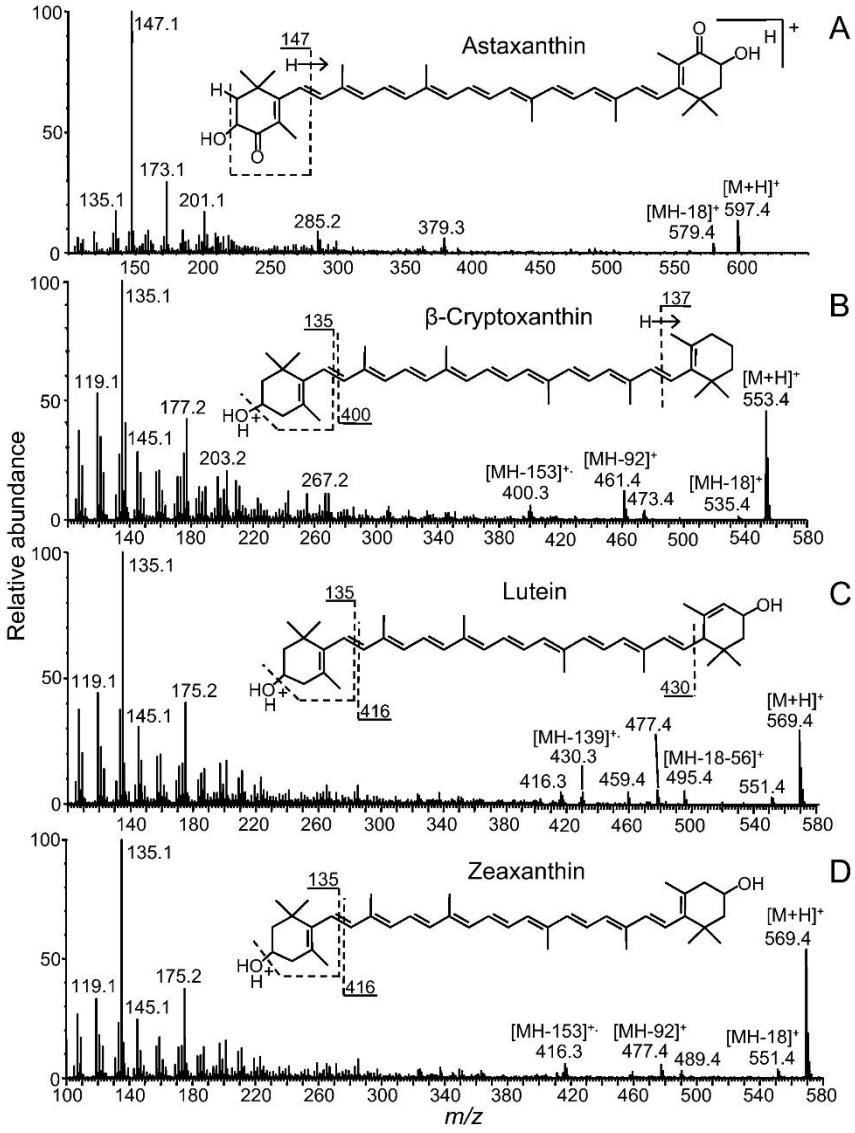

Figure 5. MS/MS spectra of xanthophylls in positive APCI: (A) astaxanthin; (B) β-cryptoxanthin; (C) lutein; and (D) zeaxanthin. Reprint from Ref. [132], with permission of Elsevier.

A comparative study between the three sources of ionization at atmospheric pressure (ESI, APCI and APPI) showed that the most powerful technique for carotenoids analysis is APCI [139]. In this study, 11 xanthophylls and four carotenes were analysed, and the effect of four dopants in APPI was evaluated. Carotenoids and carotenoid precursors observed with the strongest signal in APCI were: phytofluene, phytoene, echinenone, neoxanthin, antheraxanthin, astaxanthin, adonixanthin, zeaxanthin, β-apo-8′-carotenal, 3-hydroxyechinenone, β- and α-cryptoxanthin. ESI presented the best results for violaxanthin and lutein analysis, whereas APPI exhibited the highest level of ionization for lycopene and β-carotene. In addition, the four dopants employed (acetone, toluene, anisole, and chlorobenzene) showed increases in the signal for all carotenoids analysed in APPI. As a result, the highest sensitivity effect of dopants was observed for carotenes.

Also, the addition of organic modifiers such as ammonium acetate in the mobile phase has been reported as a determining factor to increasing the ionization yield when using the APCI source [121].

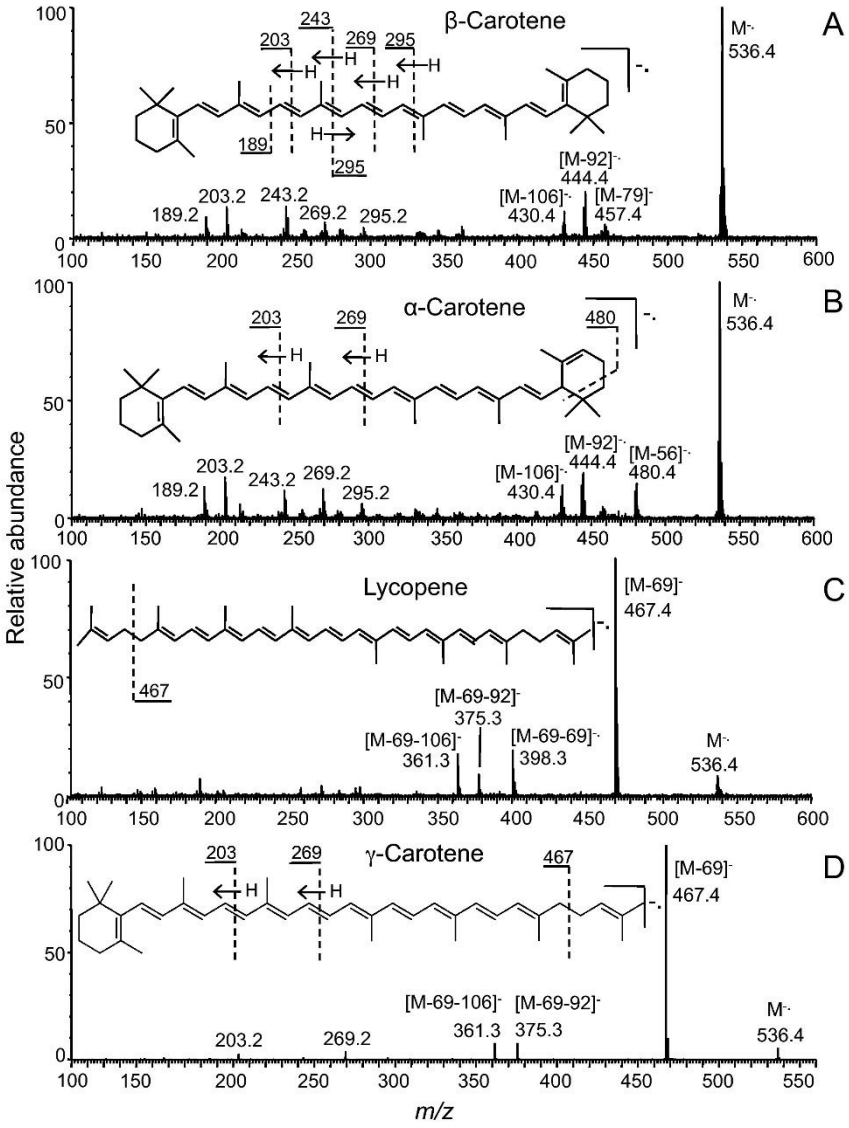

Figure 6. MS/MS spectra of carotenes in negative APCI: (A) β-carotene; (B) α-carotene; (C) lycopene; and (D) γ-carotene. Reprint from Ref. [132], with permission of Elsevier.

Once these metabolites are separated and ionized, they pass to the mass analyser. Four types of analysers are mainly used for the identification of carotenoids from bacteria (see Table 1): ion trap (IT), triple quadrupole (QqQ), quadrupole time of flight (QTOF) and Orbitrap. IT allows the structural elucidation of unknown carotenoids by successive fragmentations in low resolution tandem MS (MS^2, MS^3, MS^4…). QqQ is mainly employed for targeted and quantitative analysis due to the high sensitivity obtained operating in Multiple Reaction Monitoring (MRM) mode. QTOF and Orbitrap analysers provide crucial structural information on the basis of the exact mass both molecular ions and fragments obtained by tandem-MS in high-resolution. Table 1 summarizes some representative applications for these MS analysers.

Carotenoid extracts from bacteria very often contain high concentrations of lipids (mainly triglycerides, TAGs) that are part of the cell membranes. The two main disadvantages of TAGs are high background noise in mass spectrometry with APCI(+) source and similar fragmentation patterns to carotenoid esters [7,67]. Thus, a clean-up step

of the extracts is often required, that may involve biological methods using enzymes or physical methods using low temperatures. Although extraction and cleaning processes may seem time-consuming and cumbersome, especially the latter, they are suitable for subsequent MS identification [7,67,117].

5. Applications in Food and Textile Industries

Commercial carotenoids are mainly produced by synthesis. However, consumers' interest in products with carotenoids of natural origin has increased in recent years [32]. These compounds exhibit a wide range of applications in pharmaceutical, cosmetic, textile and food industries, among others [140,141]. In addition, the food industry has focused on manufacturing products that have striking colours of natural origin. Bacterial carotenoids applications in the food industry include the use of *Brevibacterium linens* in the fermentation of Limburger and Port-du-Salut cheeses, that produces the characteristic colour in these dairy products [96]. Likewise, a carotenoid that has shown wide versatility is astaxanthin. When this carotenoid is obtained from *Paracoccus carotinifaciens*, it which is used in the pigmentation of salmon and rainbow trout [142,143]; whereas astaxanthin from *Mycobacterium lacticola* is employed in fish feed for its antioxidant and photo-protective nature [144]. Additionally, xanthophylls such as canthaxanthin are profiteers in the pigmentation of processed foods such as margarine, butter, and confectionery. For example, canthaxanthin from *Haloferax alexandrines* increases the pigmentation of salmon flesh [145]. Also, zeaxanthin produced from *Flavobacterium sp.* is being used as an additive in poultry feed to change the colour of egg yolks and chicken skin to a darker yellow [146,147]. The industrial production of these carotenoids from bacteria such as *Dietzia sp.*, *Bradyrhizobium sp.* or *Gordonia alkanivorans* could also potentially be employed in the food industry [105,108,148].

On the other hand, the dyes used in the textile industry are largely of synthetic origin, involving highly polluting production processes. Thus, textile dyes extracted from bacteria can be a solution to this environmental problem. *Serratia marcescens* and *Janthinobacterium lividum* produce a pigment called violacein that has been shown to be an effective dye for different types of fabrics such as cotton, polyester, wool, and silk [149,150]. The desired colour intensity will depend on the time of exposure of the fabric to the bacteria and the amount of pigment that is extracted from them. Similarly, a yellow pigment from Thermomyces has been successfully used in the dyeing of silk [151,152]. *Talaromyces verruculosus* produces a red pigment that is capable to dye cotton [152]. In addition, *Vibrio spp.* Is a Gram-negative bacterium that has a bright red pigment, known as prodigiosin, which has been used to dye wool, nylon, and silk [32].

6. Conclusions and Perspectives

The great interest in the consumption of carotenoids of natural origin has encouraged the search of new sources for these compounds. Bacteria stand out as an excellent alternative due to the large variety of carotenoids that can be produced by different species. Moreover, bacteria can be considered a renewable source of carotenoids. In this paper, different methodologies frequently used for the extraction of bacterial carotenoids have been reviewed. Many of these methods involve multiple steps for extraction, concentration and resuspension of extracts, using large amounts of organic solvents such as hexane, MeOH, and $CHCl_3$, among others. Therefore, green extraction methodologies become environmentally friendly alternatives that should be considered in future research. Their use has been shown to be efficient in other microorganisms such as algae and fungi [126,127,153]. Moreover, bacterial carotenoids, very often found at low concentrations levels, makes the identification a challenging task. Therefore, the use of sensitive and high-throughput analytical methodologies such us HPLC-MS for qualitative and quantitative analysis of bacterial carotenoids becomes an essential requirement. The chromatographic separations have been developed with different stationary phases,

although C18 and C30 columns are the most commonly used. The APCI source shows the best performance in the ionization of carotenoids, regardless of the mass analyser used. Thus, HPLC-DAD-APCI-MS/MS is the technique of choice for identifying carotenoids produced by new bacterial strains, that has been reported over the last few years [154–163]. We predict a continuous development in the near future towards more sensitive and higher resolution methodologies that will allow to widen the coverage of carotenoids that can be identified in bacteria through the improvement of chromatographic columns, comprehensive methodologies in two-dimensional Liquid Chromatography (LCxLC), ionization processes and MS analysers. Finally, the search of new (or modified) bacteria as natural sources for carotenoids will remain a hot area of research for years to come.

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