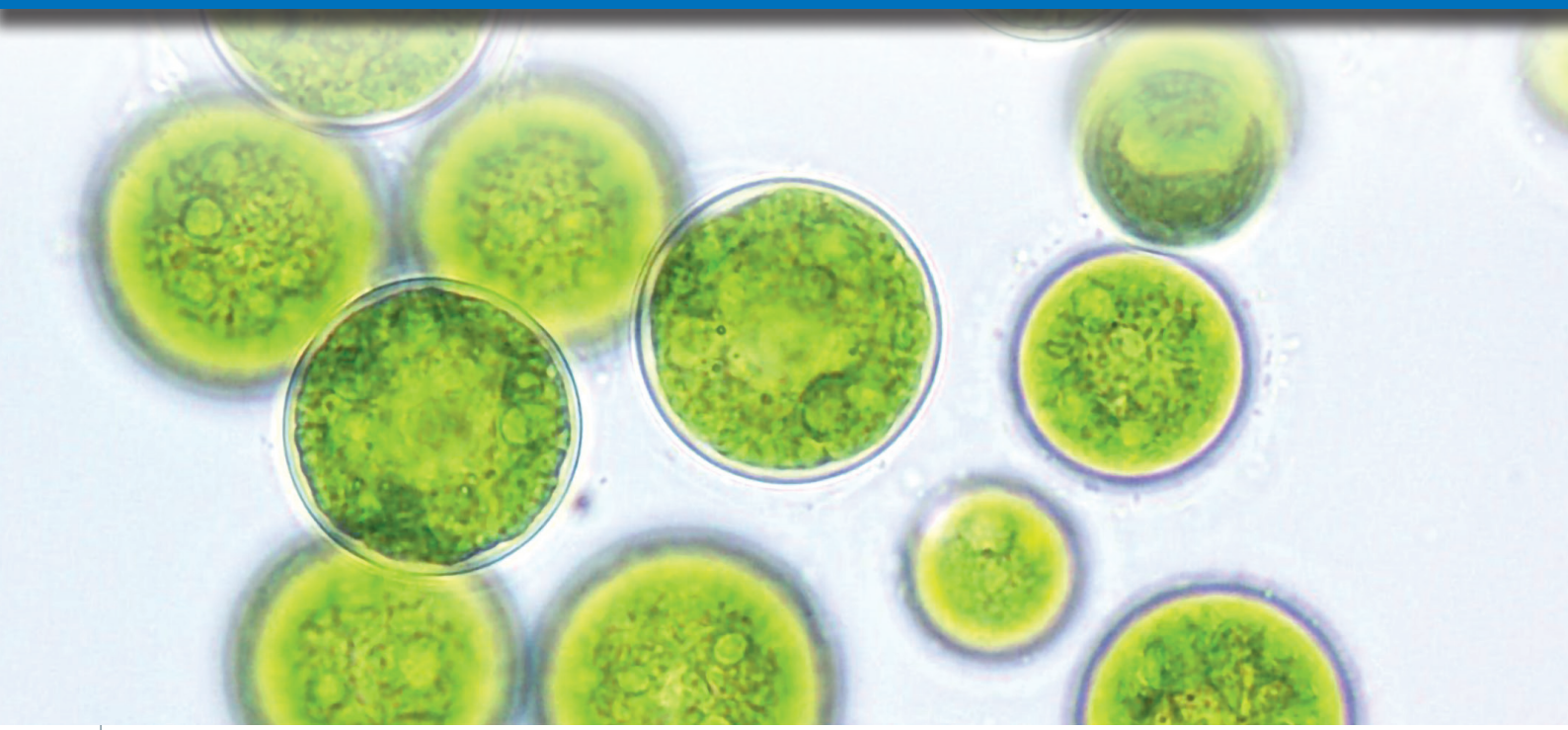

Rapid elucidation of carotenoids in microalgae formulations by MRMS aXelerate

Arthrospira platensis, better known as Spirulina, is one of the most important microalgae species. This cyanobacterium possesses a rich metabolite pattern including high amounts of natural pigments such as carotenoids.

Abstract

In this study, Spirulina pigments in three different commercial dietary supplements were characterized. Direct infusion (DI) Magnetic Resonance Mass Spectrometry (MRMS) was proven to be a fast (4 min) and very accurate (mass accuracy ≤ 0.1 ppm) tool for these kinds of studies. In this experiment 49 pigments were tentatively detected. The profile revealed different classes of metabolites such as carotenes, xanthophylls and chlorophylls.

Introduction

Among the natural matrices rich in bioactive compounds, microalgae represent one of the most promising matrices [1]. These microorganisms are a source of various biologically active molecules, including amino acids,

Keywords: MRMS, microalgae, direct infusion, metabolomics

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polyunsaturated fatty acids, minerals, proteins and pigments. *Arthrospira platensis*, also known as Spirulina, is one of the most economically important species. Spirulina-based products are used by athletes as anti-fatigue and amino acid supply, and for their anti-aging detoxifying and antioxidant properties in cosmetics. The antioxidant potential of Spirulina is partially attributed to the high content of natural pigments, especially carotenoids, which are also recognized for having numerous healthy benefits. Despite the success of Spirulina in the market, the profiling of pigment in this species has been only partially described. The determination of carotenoids in Spirulina has been carried out mainly by liquid chromatography (LC) coupled with diode array detector (DAD) and mass spectrometry (MS) detection by employing a low-resolution mass analyzer [2]. Recently high-performance thin layer chromatography (HPTLC) was used for the identification of carotenoids in Spirulina [3]. Given that the Spirulina pigment fraction is highly complex, conventional LC-MS based methods suffer from low separation efficiency of very complex mixtures as well as long analysis time and low mass accuracy, which can result in inaccurate and incorrect compound identification. In this regard, the objective of this study was the development of a combined platform for qualitative and quantitative characterization of Spirulina pigments in different dietary supplements. To tackle this task, we exploited the very accurate mass measurement and ultra-high mass resolution of Magnetic Resonance Mass Spectrometry (MRMS) for the qualitative profiling of the extract.

Methods

Chemicals

LC-MS grade acetonitrile, methanol, and standards of β-carotene, lutein, and zeaxanthin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Spirulina powders and tablets were purchased from FarmaLabor SRL (Canosa di Puglia, Barletta-Trani, Italy) and Dr. Giorgini (Bologna, Italy) respectively. Lab-made Spirulina powder was kindly donated by a local farmer.

Sample Extraction

Pigment extraction was carried out as follows. 350 mg of Spirulina powder (tablets were prior pulverized in a mortar) were treated with 50 mL of ethanol fortified with 20 µg/mL of Butyl hydroxyl toluene (BHT) to prevent oxidation. The sample was subjected for 15 min in an ultrasonic bath, then the suspension was stirred for 30 min at room temperature and then centrifuged for 10 min at 6000 rpm at room temperature. The supernatant was removed, and the pellet was retreated following the same protocol another four times. Finally, the supernatants were pooled and lyophilized. The same conditions were employed for each sample of Spirulina.

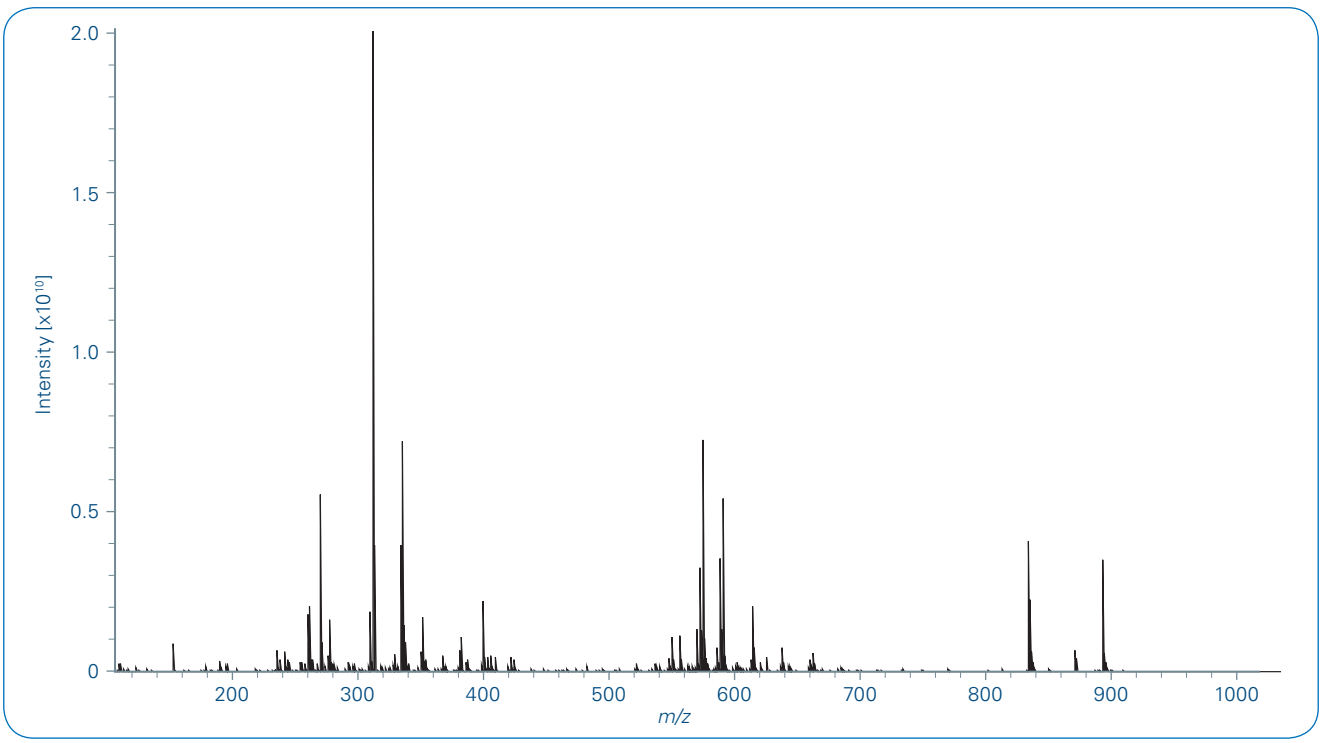

Figure 1: Broad band APCI-MRMS spectrum of a Spirulina powder extract

MS analysis

Data was acquired on a solariX XR 7T MRMS (Bruker Daltonik GmbH, Bremen, Germany) equipped with an Apollo II APCI ion source. The instrument was tuned and externally calibrated with a standard solution (100 µg/ml in 50% acetonitrile) of sodium trifluoro-acetate (NaTFA). Samples (10 µg/mL in methanol) were infused into the ion source at 50 µL/min. Mass spectra were recorded in broadband mode in the range 150–3000 m/z with an ion accumulation time of 20 ms. 200 single scans were added. Spectra were acquired with 8 million data points (8M) resulting in a mass resolution of 700,000 at *m/z* 400 with a transient length of 6 seconds. Nebulizing (N_2) and drying gases (N_2) were set at 1 and 4 L/min, respectively. Drying gas temperature was set to 200°C. Five measurement replicates were carried out of each sample.

Data processing

Peak alignment and tentative identification of compounds was based on accurate mass measurement. Spectra were loaded in MetaboScape 4.0 (Bruker Daltonik GmbH, Bremen, Germany) for feature extraction and database search.

Results

MRMS is characterized by unmatched ultra-high mass accuracy and mass resolution, which are ideal for the analysis of complex mixtures such as phytochemical samples. In this approach, we employed APCI ionization, which outperformed electrospray for almost all analytes classes (data not shown). Table 1 shows the detection and tentative identification of compounds in three formulations. A high number of tentatively identified compounds could be detected with respect to previous investigations of Spirulina [2].

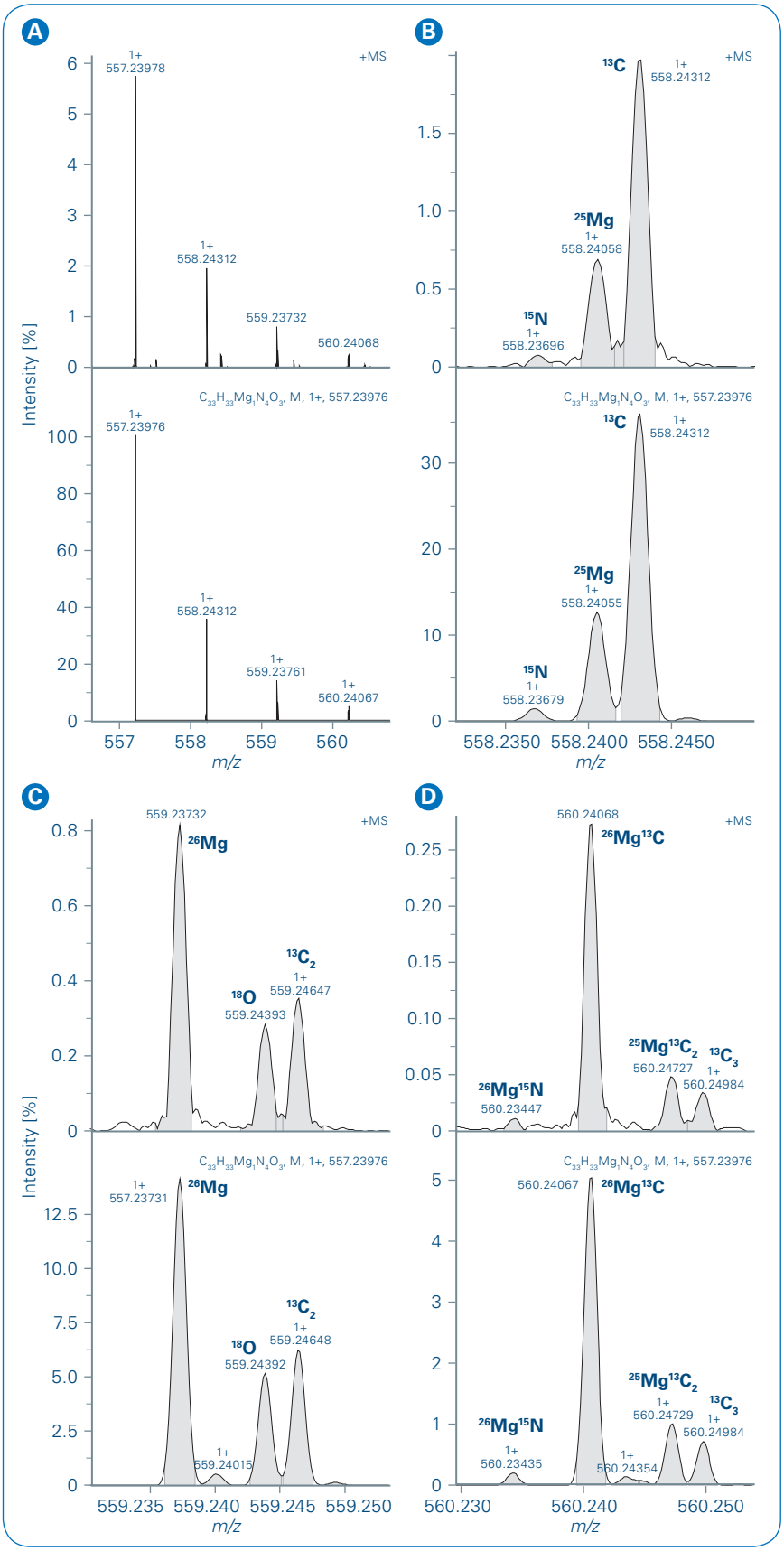

Figure 2: Zoom-in of broad band spectrum at m/z 557 of compound Pyro Chlorophyllide-a (Pyro Chld a) ($C_{33}H_{32}MgN_4O_3$) and IFS pattern. Measured spectrum in top and simulated spectrum in bottom, (A) zoom-in at m/z 557 of isotopic pattern, (B) A+1 pattern, (C) A+2 pattern, (D) A+3 pattern.

Figure 1 reports the full APCI spectrum of a Spriulina extract. Different carotenoid classes could be identified: hydroxyl, epoxy, keto-carotenoids, carotenes as well as chlorophylls and pyro chlorophylls. Several compounds are reported here for the first time present in Spirulina (Table 1). Ultra-high mass accuracy lead to highly confident identification, such as for the Pyro Chlorophyllide-a, a chlorophyll derivative (Figure 2). The molecular formula could be confirmed by isotopic fine structure (IFS) of the A+1, A+2 and A+3 pattern shown in Figure 2 B-D. Based on the identified molecular formula possible structures of this molecule can be found using CompoundCrawler (Figure 3). A further benefit of DI-MRMS is the analysis time. A mass spectrum could be acquired in only four minutes which is much faster than conventional LC-MS experiments for detection of carotenoids.

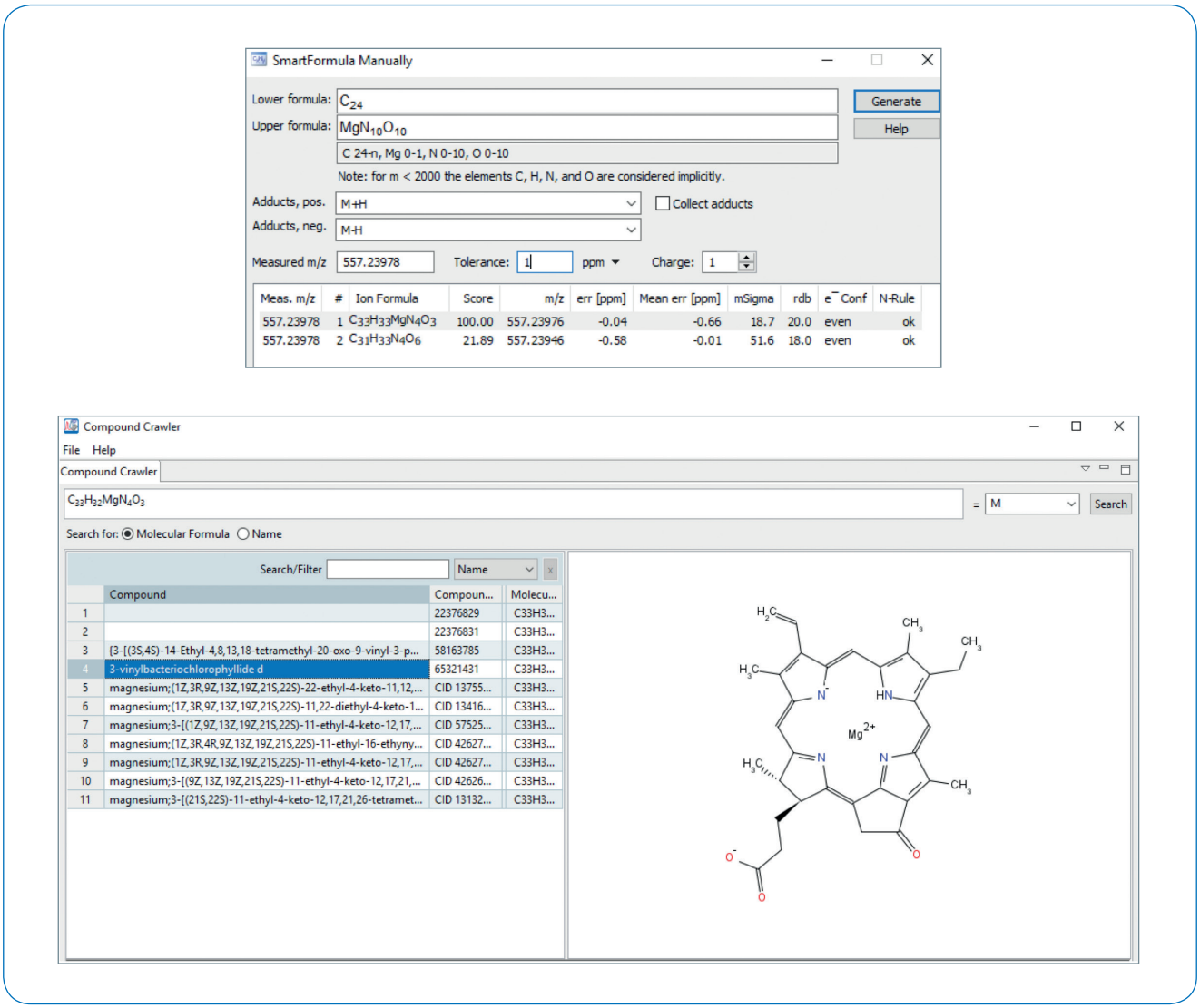

Figure 3: Accurate mass combined with isotopic fine structure (IFS) results in top score for molecular formula of $C_{33}H_{33}MgN_4O_3$. Possible structures have been found for this molecular formula by CompoundCrawler.

Compound	Molecular Formula	$[M+H]^+$ DI-APCI-MRMs	Mass accuracy
Apo-12-Violaxanthal[1]	$C_{25}H_{34}O_3$	383.25809	-0.05
Vaucheriaxanthin[1]	$C_{40}H_{56}O_5$	617.42010	-0.08
Diadinoxanthin[1]	$C_{40}H_{54}O_3$	583.41458	-0.02
Canthaxanthin	$C_{40}H_{52}O_2$	565.40402	-0.01
Ethyl β-apo-8′-carotenoate[1]	$C_{32}H_{44}O_2$	461.34143	-0.04
Adonirubin[1]	$C_{40}H_{52}O_3$	581.39892	0.01
Diatoxanthin[1]	$C_{40}H_{54}O_2$	567.41967	-0.02
β-Apo-8′-carotenal[1]	$C_{30}H_{40}O$	417.3152	-0.01
Hexadehydro-β,β-caroten-3-ol[1]	$C_{40}H_{50}O$	547.39347	-0.06
Rhodoxanthina	$C_{40}H_{50}O_2$	563.38838	-0.04
Astaxanthin	$C_{40}H_{52}O_4$	597.39382	0.02
Antheraxanthin[1]	$C_{40}H_{56}O_3$	585.43023	-0.02
Myxoxanthophyll	$C_{46}H_{66}O_7$	731.48807	0.08
Zeaxanthin	$C_{40}H_{56}O_2$	569.43529	0.02
10-Apo-β-carotenal[1]	$C_{27}H_{36}O$	377.28389	-0.06
α-tocopherol	$C_{29}H_{50}O_2$	431.38835	0.01
Echinenone	$C_{40}H_{54}O$	551.42473	0.02
Pyro Chl *b*	$C_{53}H_{68}MgN_4O_4$	849.51640	-0.03
Phy *a* derivate	$C_{55}H_{72}N_4O_5$	869.55755	0.01
Chld *b*	$C_{35}H_{32}MgN_4O_6$	629.24050	0.01
Chl *b*	$C_{55}H_{70}MgN_4O_6$	907.55824	0.01
Pyro Chl *a*	$C_{53}H_{70}MgN_4O_3$	835.53711	0.01
Pyro Chld *a*	$C_{33}H_{32}MgN_4O_3$	557.23978	-0.04
Pyro Chld *b*	$C_{33}H_{30}MgN_4O_4$	571.21901	0.02
OH-Chl *a*	$C_{55}H_{72}MgN_4O_6$	909.53746	0.05
Protochld *a*	$C_{35}H_{32}MgN_4O_5$	613.22959	-0.01
13-OH-Chld *a*	$C_{35}H_{34}MgN_4O_6$	631.24015	0.01
Divinyl Chl *a*	$C_{55}H_{70}MgN_4O_5$	891.52691	0.04
Chl *a*	$C_{55}H_{72}MgN_4O_5$	893.54262	-0.03
Cryptoxanthin*	$C_{40}H_{56}O$	553.44040	0.01
Chld *a*	$C_{35}H_{34}MgN_4O_5$	615.24526	-0.04
Phy *b*	$C_{55}H_{72}N_4O_6$	885.55233	0.14
15-OH-Lactone-Chl *a*	$C_{55}H_{73}MgN_4O_7$	925.53199	0.47
Pyro Pheo *b*	$C_{33}H_{32}N_4O_4$	549.24967	-0.08
15-OH-Lactone-Phy *a*	$C_{55}H_{73}N_4O_7$	903.56328	-0.28
Chlorobactene	$C_{40}H_{52}$	533.41416	0.03
Chl *a* derivate I	$C_{55}H_{68}MgN_4O_5$	889.51122	0.08
Phytoene	$C_{40}H_{64}$	545.50810	-0.03
13-OH-Pheo *a*	$C_{35}H_{36}N_4O_6$	609.27078	-0.02
OH-Phy *a*	$C_{55}H_{73}N_4O_6$	887.56810	0.01
β-carotene	$C_{40}H_{56}$	537.44547	0.01
Octadehydro-β,β-carotene	$C_{40}H_{48}$	529.38288	0.03
Phy *a*	$C_{55}H_{74}N_4O_5$	871.57318	0.02
Pheophorbide *a*	$C_{35}H_{36}N_4O_5$	593.27583	0.02
Pyro Pheo *a*	$C_{33}H_{34}N_4O_3$	535.27037	0.01
Pyro Phy *a*	$C_{53}H_{72}N_4O_3$	813.56769	0.04
δ-tocopherol	$C_{27}H_{46}O_2$	403.35706	0.01
γ-tocopherol	$C_{28}H_{48}O_2$	417.37270	0.02
Phytofluene	$C_{40}H_{62}$	543.49242	0.01

Table 1. Detected compounds found in three Spriulina formulation extracts

Conclusion

The developed analytical strategy using APCI combined with DI-MRMS has been proven for analysis of carotenoids in Spirulina pigment fractions. This method based on ultra-high mass resolution and accurate mass as well as isotopic fine structure is a promising tool for in-depth profiling of microalgae pigments. Furthermore, this study confirms the high importance of DI-MRMS for bio-compound detection such as carotenoids in Spirulina, and its importance in the nutraceutical and pharmaceutical area.

Acknowledgement

We kindly would like to thank a local farmer for providing the Spirulina powder for this study.

[1] Detected for the first time in Spirulina (*Arthrospira Platensis*). Chld: chlorophyllide; Pheo: pheophorbide; Phy: Pheophytin; Chl: Chlorophyll.

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