FONDAZIONE EDMUND MACH

AlgaeCeuticals

Algae Metabolomics

Stefan Martens

Edmund Mach Foundation – San Michele all‘Adige

founded on 12. January 1874 (IASMA); 2009 (FEM)

three separate centers: academic, **experimental** and technical assistance

Research and Innovation Centre (CRI) – first ONE HEALTH Institute in Italy

Aims:

- promoting cultural and socio-economic growth in the agricultural sector
- developing the forestry and agro-alimentary systems
- four Departments and one Transversal Structure
    - **Genomics and Biology of Fruit Crop**
    - **Food Quality Nutrition & Health**
    - Sustainable Agro-Ecosystems and Bioresources
    - Biodiversity and Molecular Ecology

} Computional Biology

UNIT METABOLOMIC - DQAN

Food quality
Fulvio Mattivi

METABOLOMIC UNIT

Biotechnology of natural products
Stefan Martens

Metabolomics
Urska Vrhovsek

Nano HPLC (Dionex)-Orbitrap FTMS (Thermoscientific)

Acquity UPLC-MS/MS Xevo (Waters)

Acquity UPLC - Synapt Q-TOF HDMS (Waters)

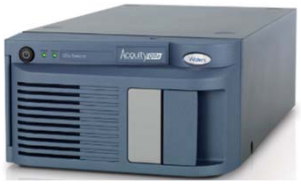
More information at www.fmach.it

FEM – Metabolomics platform













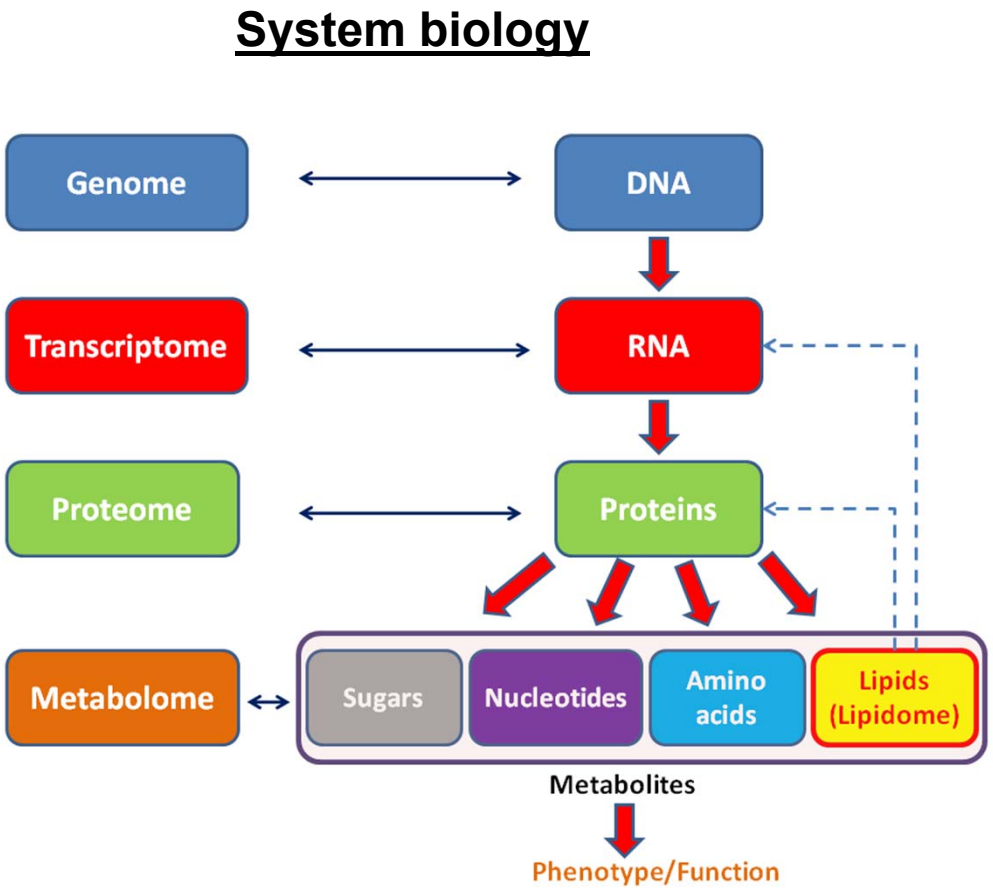
System Biology & sequence of *-omics*

What can the cell potentially do?

What is currently being turned on?

What enzymes are currently active?

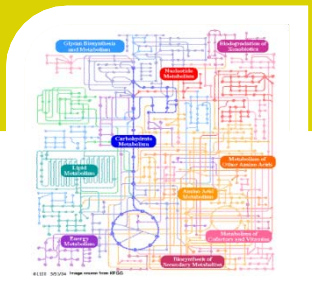
What is being produced/consumed?



Metabolomics - definition

- Metabolomics is an analytical technique which ideally measure **all** metabolites in plant, human, animal, microorganisms, - the analysis of the **whole** metabolome (known and unknown compounds) - which are the final **products** of gene expression
- final objective of Metabolomics is to come closer to this ideal
- Metabolomics is a complementary science to transcriptomics and proteomics
- Multidisciplinary: chemistry + biology + physics + mathematics + informat
- Metabolomics plays a significant role in bridging the phenotype-genotype gap linking each gene to its final product.

Metabolome – definition



Metabolome:

all organic compounds of the specific plant or organism (vitamins, amino acids, antioxidants, hormons, sugars, aromatic compounds, …)

How big is metabolome:

- microorganisms > 600 metabolites
- human > 2.500 metabolites + food + drugs, ...
- **plants 200.000 metabolites, per species 5.000-10.000**

Known compounds:

- grape, apple: estimate 5.000-10.000 metabolites, **known ca. 10%**
- human diagnostics: 2% of endogenous metabolites

Metabolomics - definition

Mass Spectrometry (MS)

Direct infusion/Imaging → few hundreds metabolites

Gas Chromatography (GC) → few hundreds metabolites → GCxGC / Derivatisation

Liquid Chromatography (LC) → few thousands metabolites → Reverse Phase / Normal Phase

Capillary Electrophoresis (EC) → few hundreds metabolites → ESI- / ESI+

Nuclear Magnetic Resonance (NMR) → NMR: up to 100 metabolites

Targeted analysis

The current knowledge of chemical processes in plants, animals and humans are mainly based on conventional studies in which profiles of metabolites involve "targeted" metabolites or "targeted" classes of metabolites.

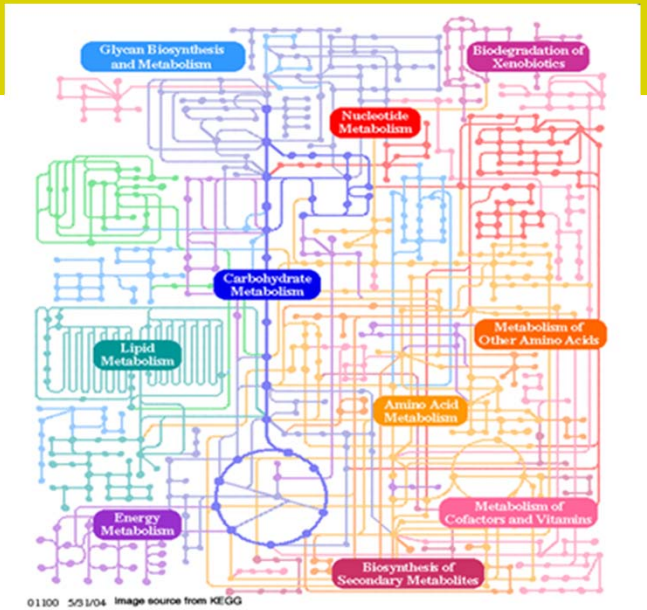
As a consequence the majority (80-90 %) of plant metabolites remain unknown.

T*argeted* versus *Untargeted* analysis

Prefer targeted methods when:

- ❑ you focus in a targeted group or single metabolite (you know what to measure)
- ❑ you want/need the absolute concentration
- ❑ you don't know very good your **instrument** (analytical chemistry skills)
- ❑ you are not familiar with compound annotation
- ❑ you have a poor knowledge of your **sample metabolome**
- ❑ you don't have plenty of **time** for data analysis / you want fast results
- ❑ you want to work "alone" (biology, organic chemistry, biochemistry, analytical chemistry, bioinformatics, chemo-metrics)

Metabolomics

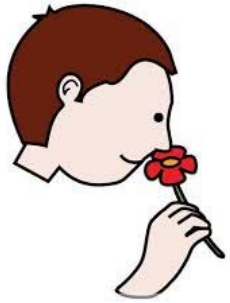


For future perspectives

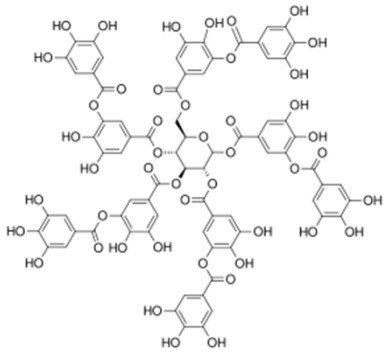
Metabolomics is a powerful tool through which alterations in diverse metabolic pathways could be better understood.

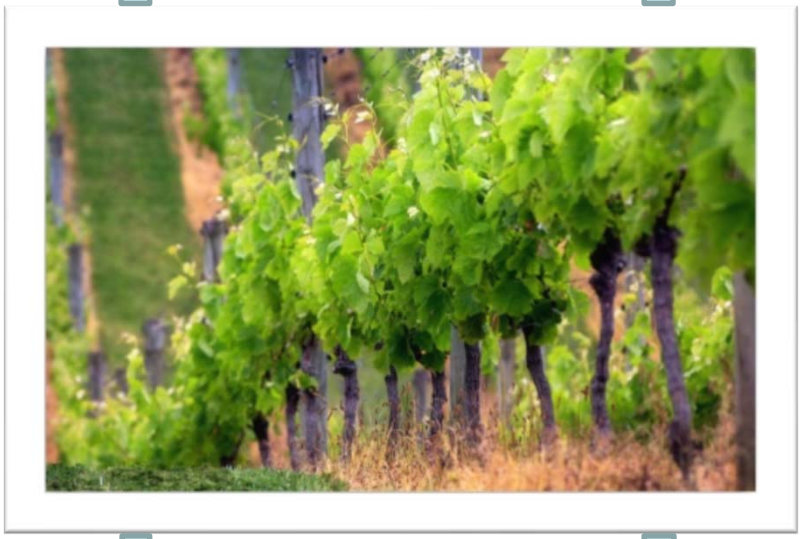
Chemical classes of metabolites

Volatile compounds



Secondary metabolites





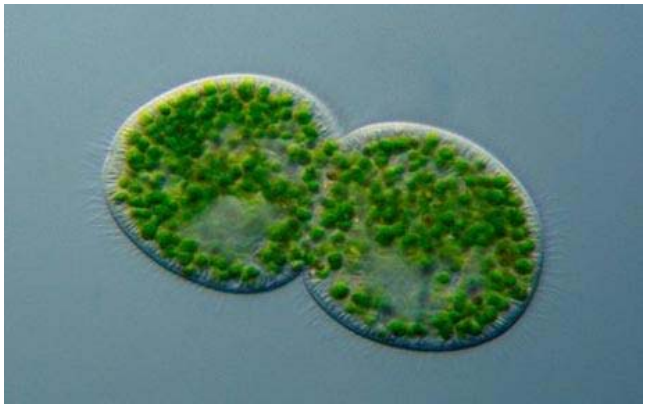
Primary metabolites

Lipids

Algae

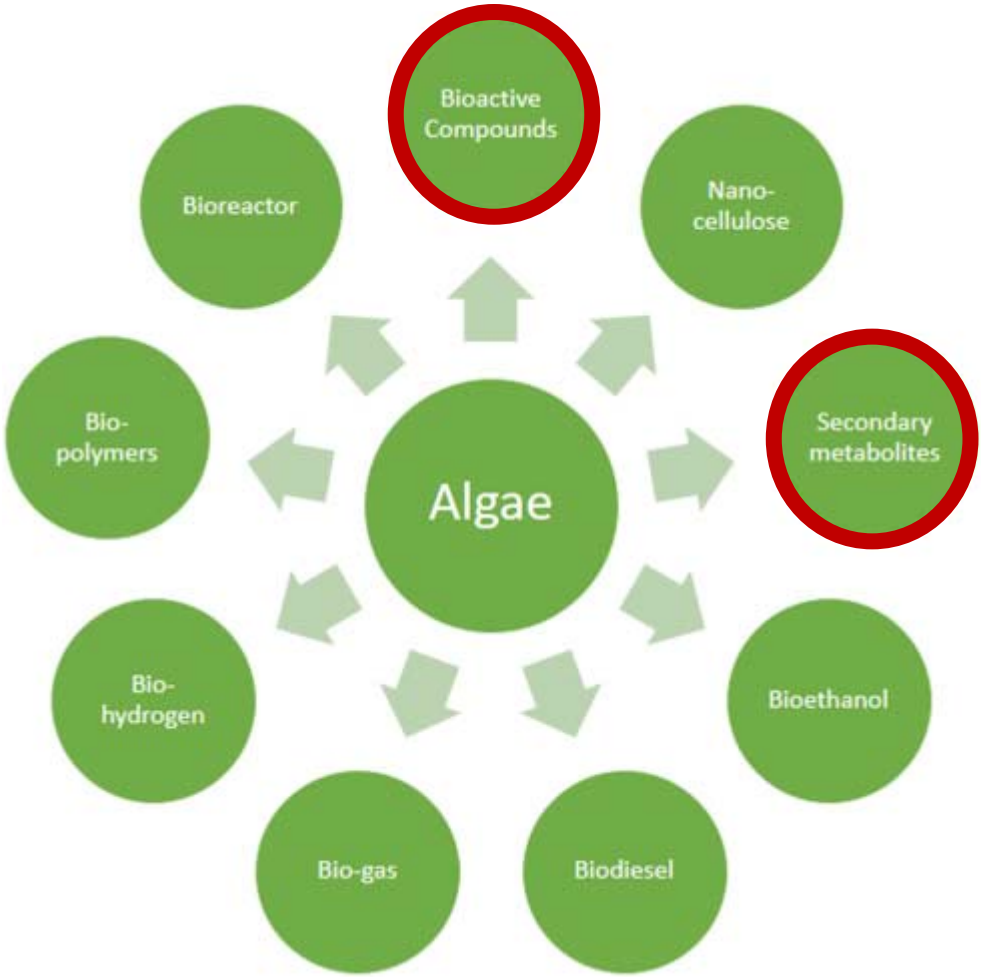
- No definition of algae is generally accepted.

- simple, non-flowering, and typically aquatic eukaryotic organisms
- ranging from microscopic single-celled forms to multicellular forms 100 feet (30 meters) or more long
- large group
- algae contain chlorophyll but lack true stems, roots, leaves, and vascular tissue.
- classified into the six phyla
  - Euglenophyta,
  - Crysophyta,
  - Pyrrophyta,
  - Chlorophyta,
  - Phaeophyta,
  - Rhodophyta



- cyanobacteria are often referred to as "blue-green algae", but most authorities exclude all prokaryotes from the definition of algae!!

Algae applications



Alassali et al., 2013

Metabolite analysis in Algae

- growing interest in algae derived products
- high quality
- different applications
  - food, nutraceuticals, phytopharmaca, cell factories
- (poly)phenols, carotenoids, terpenoids and others


Photo by Alexander Hafemann on Unsplash

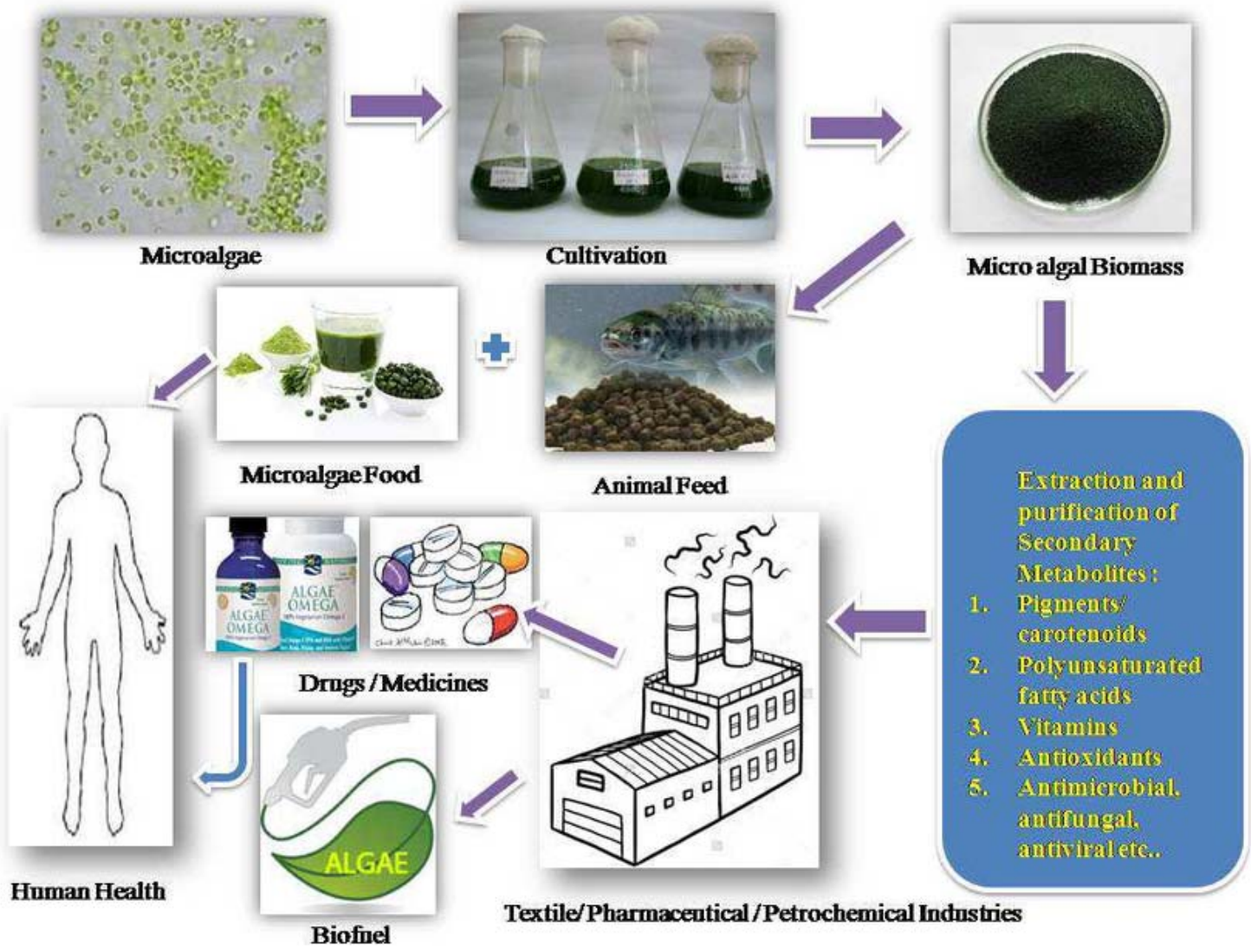
- main focus on the major and/or active compounds/indication

➔ develop robust, reliable and fast tools for quality assessment

➔ use metabolite analysis/fingerprints to develop more detailed metabolite pattern and pathway schemes

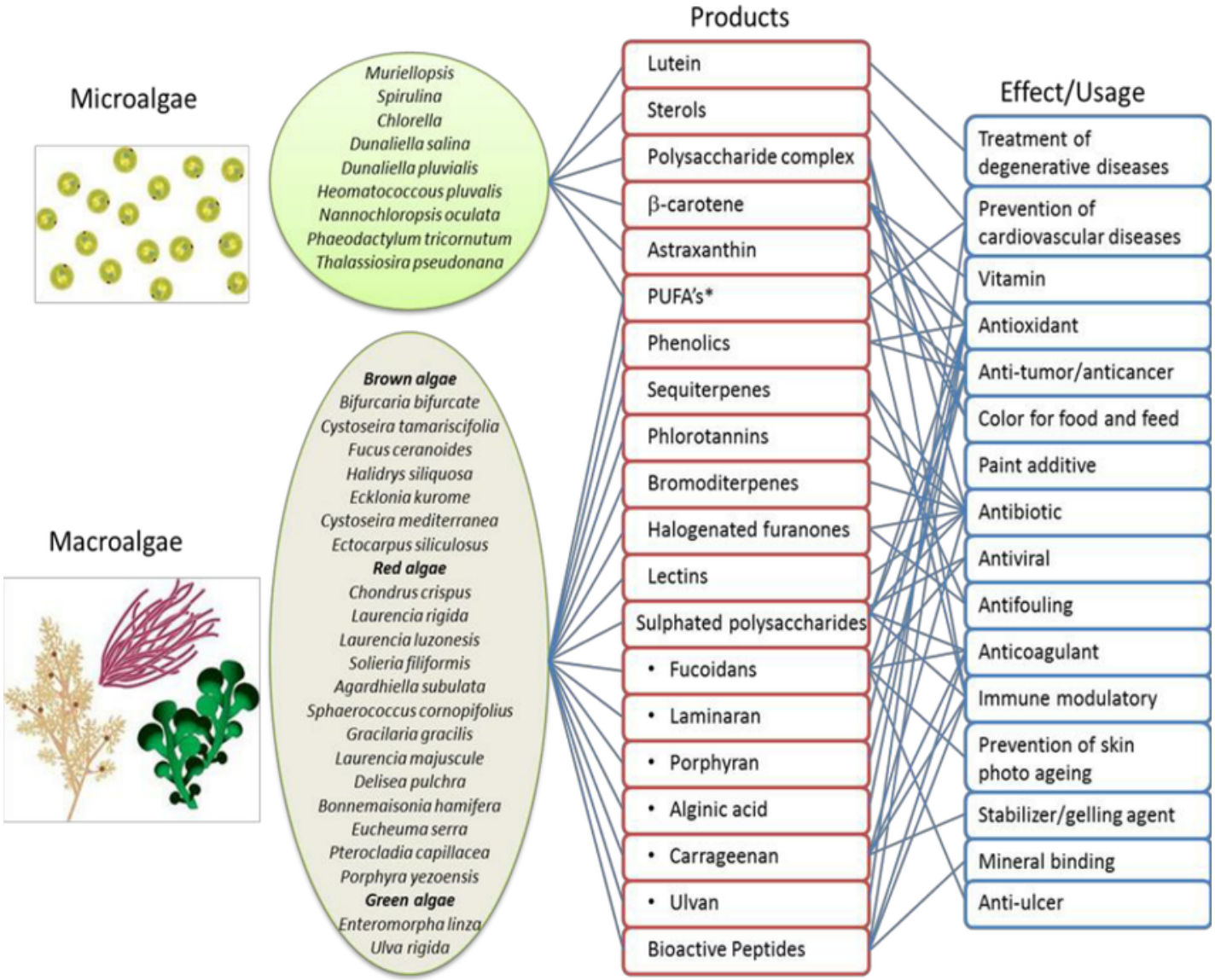
➔ improve selection and cultivation strategies

From cultivation to application



Sigamani & Natarajan, 2016

Algae metabolites

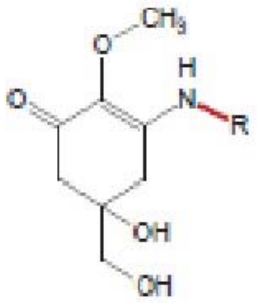


Alassali et al., 2013

Mycosporin-like Amino Acids (MAA) from microalgae as possible sun-screens

The ability of microalgae in producing MAA can been tested in microalgal coltures according with the following workflow

General structure of MAA



MAA are secondary metabolites produced by different organisms that protect cells from UV-induced damages. They are characterized by very high absorption between 310 and 360 nm.

Solvent extraction

High absorbance in the UV region reflects the presence of highly UV-absorbing compounds.

HPLC analysis (UV detection)

Identification of highly-UV absorbing fractions

HPLC analysis (MS detection)

Determination of molecular masses and fragmentation pattern of highly UV-absorbing compounds

Database search

Identification of the highly UV-absorbing compounds (if present in databases)

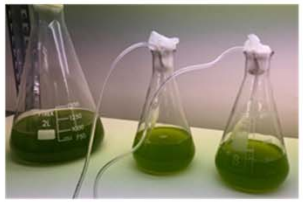
Purification and further analysis

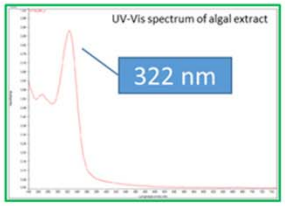
If the compounds are not reported in database, High-Resolution MS and NMR are needed for structure elucidation

Mycosporine-like aminoacids (MAA) from microalgae

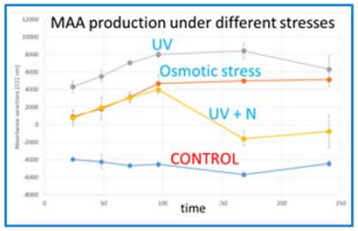
optimization of growth conditions, extraction process and analytical procedure

A *Chlorella* strain was identified as a good source of MAA from a survey conducted on different FEM microalgae strains.

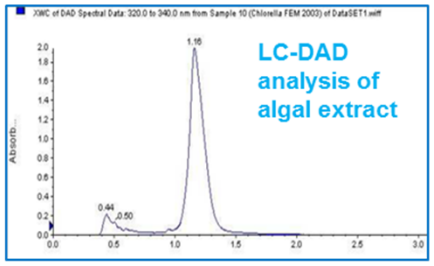


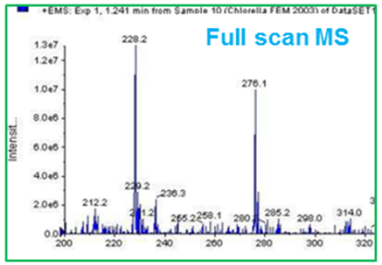


The production of MAA was stimulated by stress condition (either UV irradiation or osmotic stress), as revealed by specific growth tests.



Chemical analysis (LC-DAD and LC-MS) revealed the presence of a major compound with a high molar absorption at 322 nm and a molecular mass of 275 Da. Not present in databases.

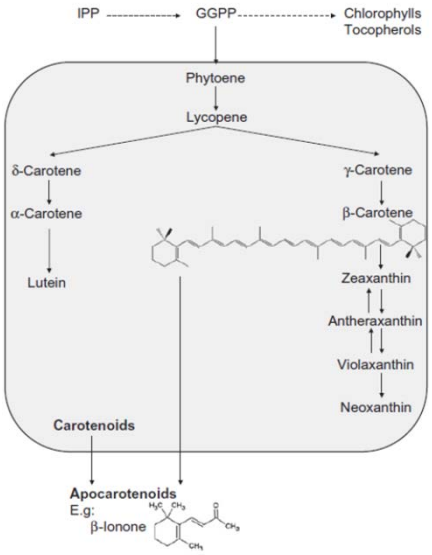


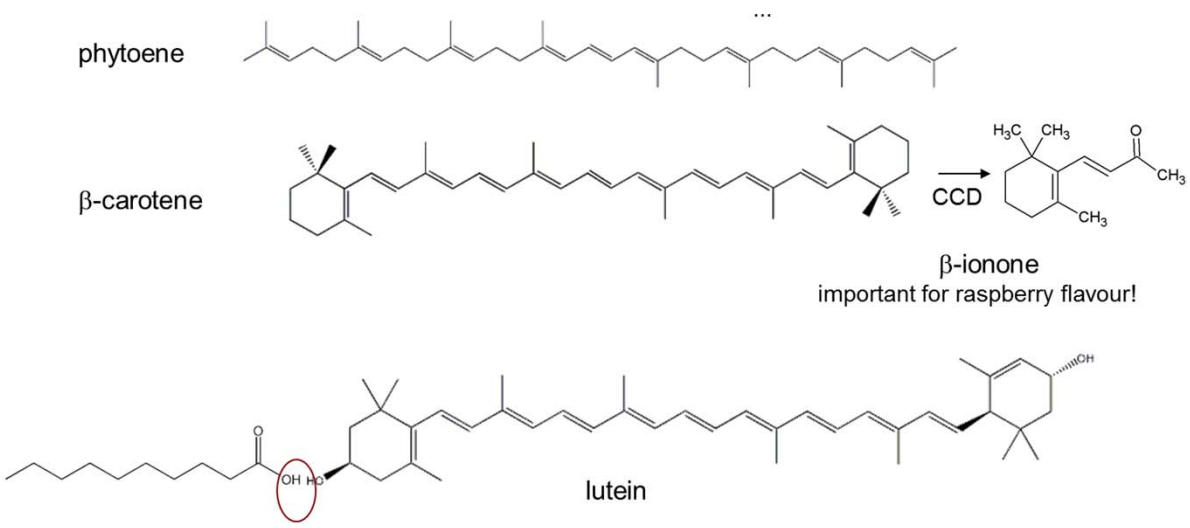


Purification efforts are in progress for isolating enough material for structural elucidation (high resolution MS and NMR).

Carotenoids: functions

- Plants & algae
  - percursors of apocarotenoids (volatiles, hormones, etc)
  - pigments
  - functions in photosynthesis
- nutrition and health:
  - provitamin A
  - macular degeneration
  - ...





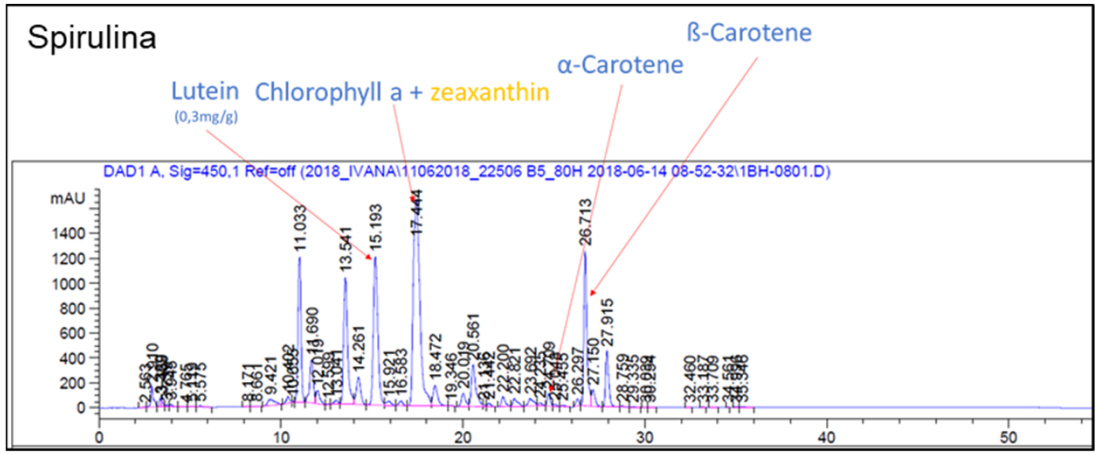
UPLC-DAD analysis of algae carotenoids

- different extraction protocols
  - solvents – MeOH, acetone, ethanol, „Rubus“ method (MeOH/Chloroform)
  - plus/minus ultrasonic, vortexen, saponification
- different analytical methods
  - 15 to 60 min, gradient
  - often highly volatile solvents → technical problems
  - overlapping peaks → R Alcace → might too complicated

→ establish solid method for carotenoid identification and quantifcation

modified from Van Heukelem & Thomas, 2001

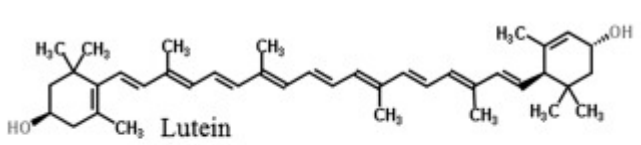
gradient, 45 min, C8 column, buffered methanol

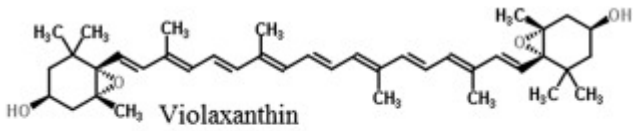


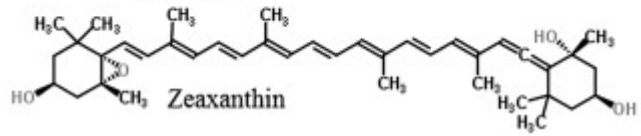
Carotenoid & chlorophyll standard collection

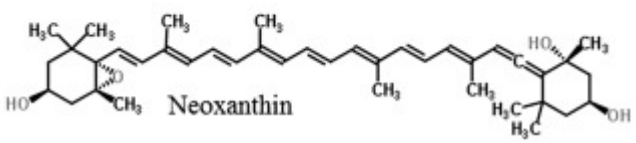
Analytcal standards

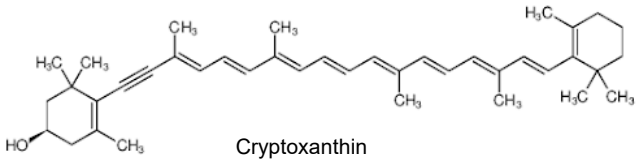
- **Neoxanthin**
- **Violaxanthin**
- **Anteraxanthin**
- **Lutein Epoxide**
- **Zeaxanthin**
- **Lutein**
- **Chlorophyll b**
- **Cryptoxanthin**
- **Chlorophyll a**
- **α-Carotene**
- **β-Carotene**
- **Phytoene**
- **Chlorophyllide a**
- **Pheophorbide a**
- **Fucoxanthin**
- **Myxoxantophyll**
- **Cantaxanthin**
- **Pheophytin a**



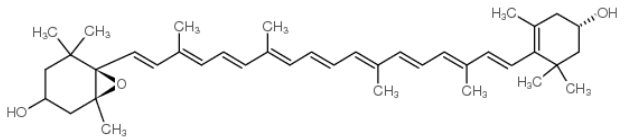




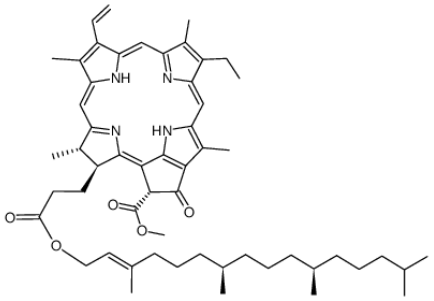




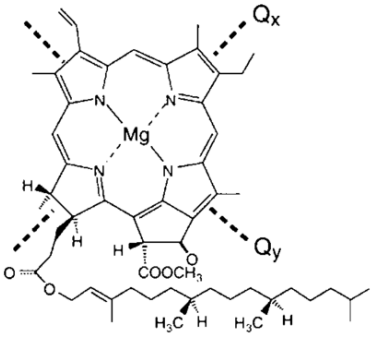
Cryptoxanthin

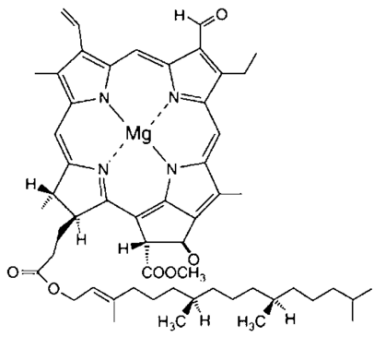


Antheraxanthin



Pheophytin-a

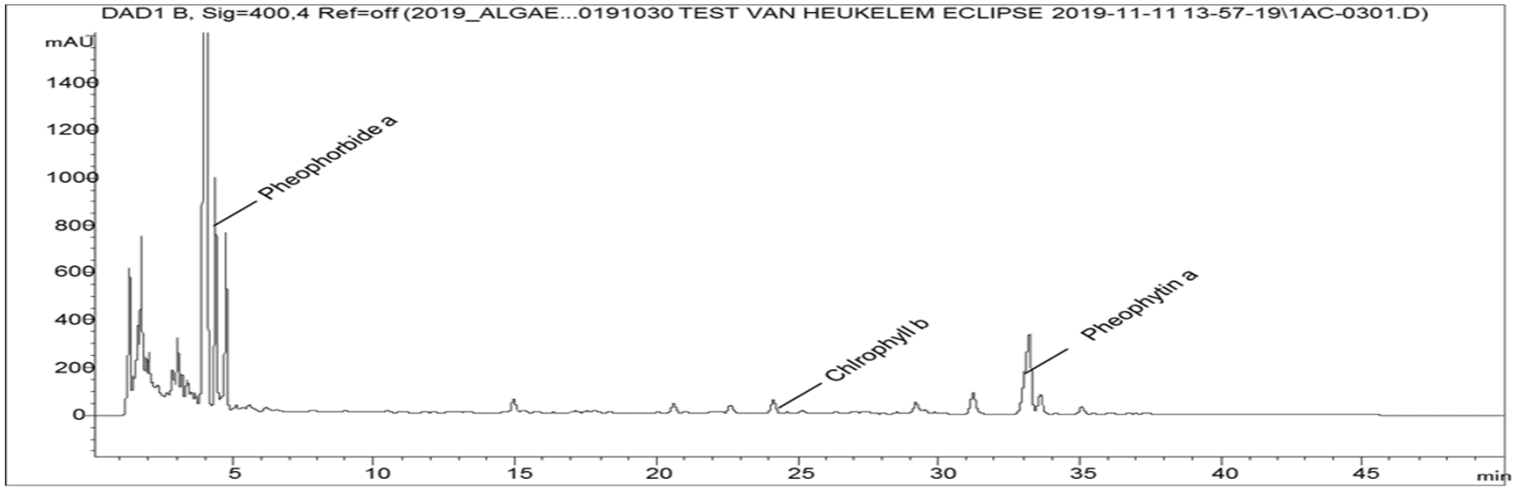




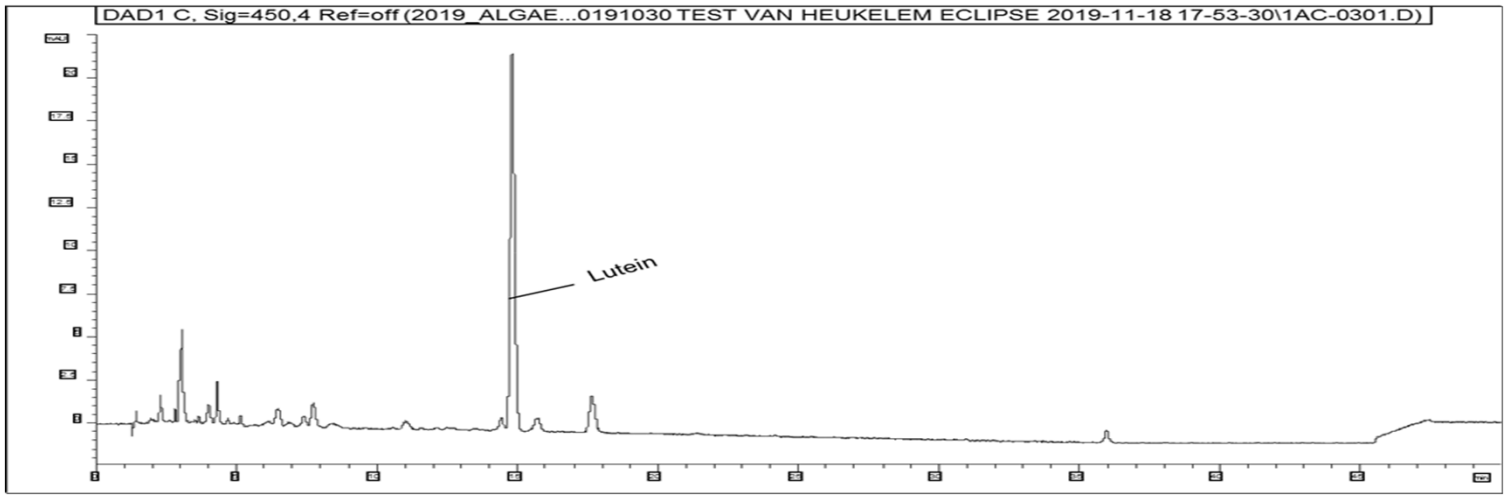
Chlorophyll a and chlorophyll b

Extraction of carotenoids and chloropfyll derivatives

Chlorella



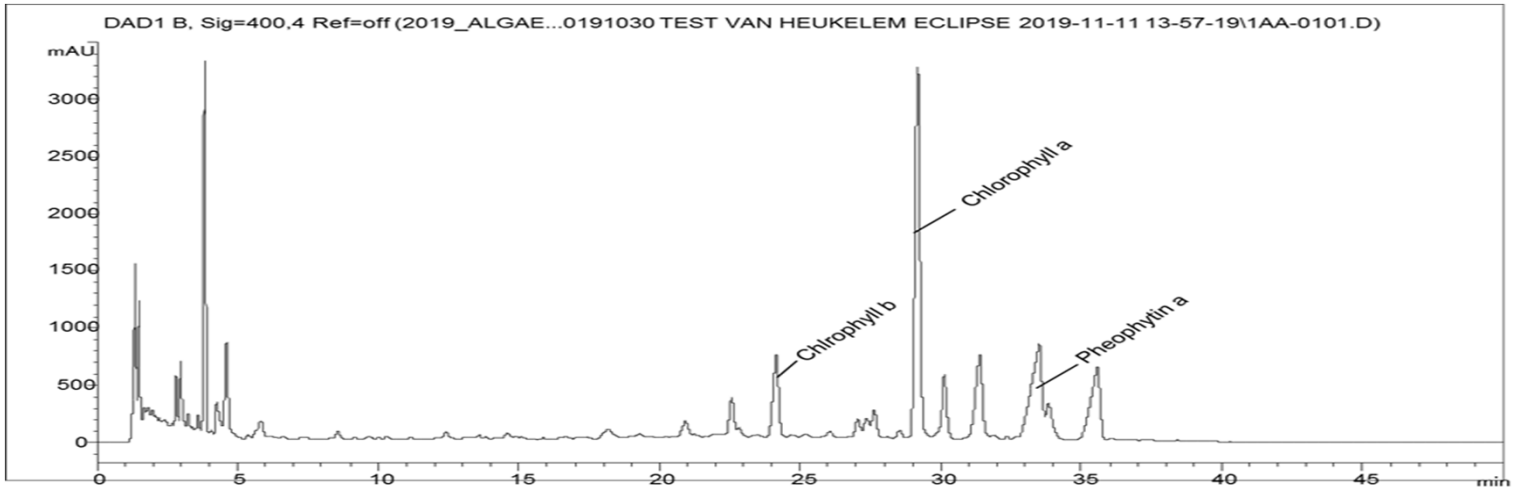
MeOH



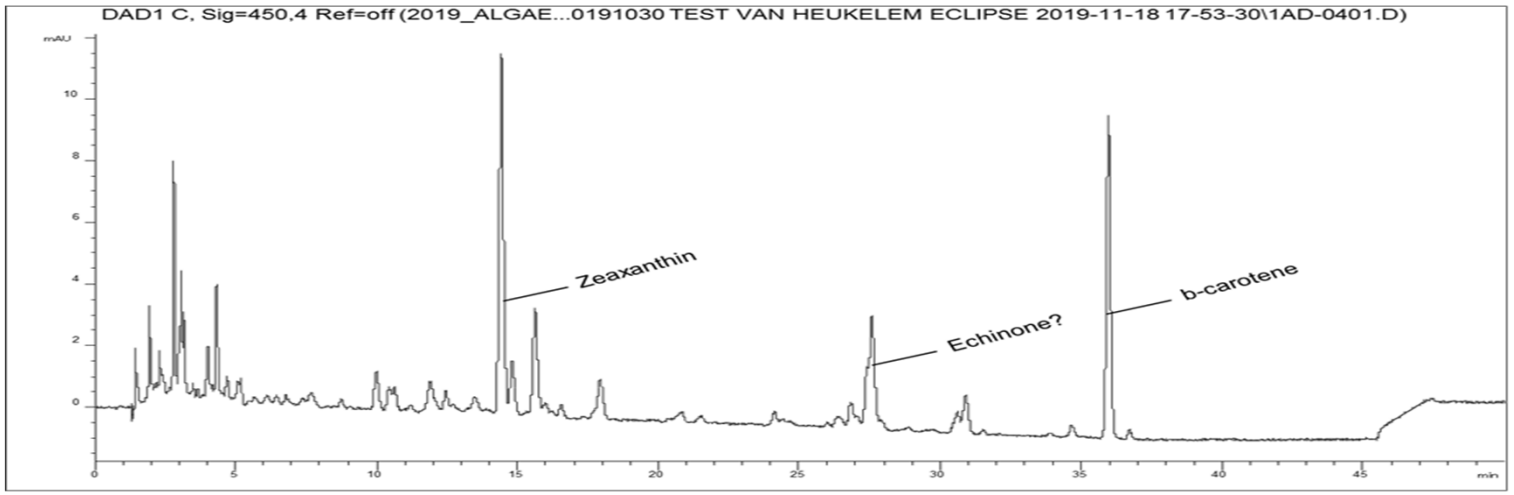
Hexane

Extraction of carotenoids and chloropfyll derivatives

Nannochloropsis



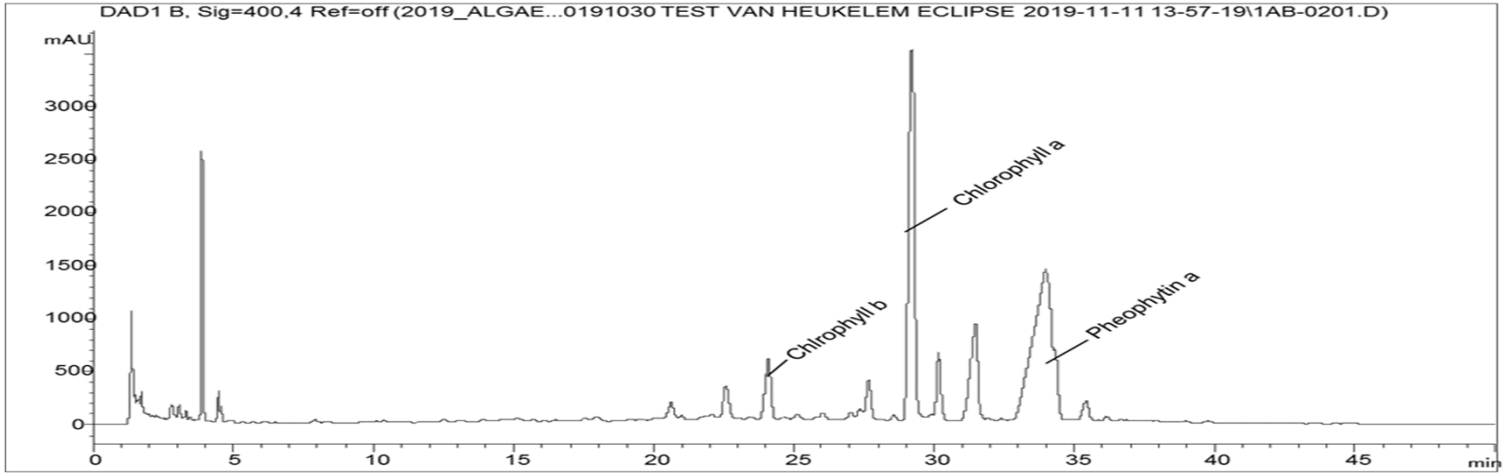
MeOH



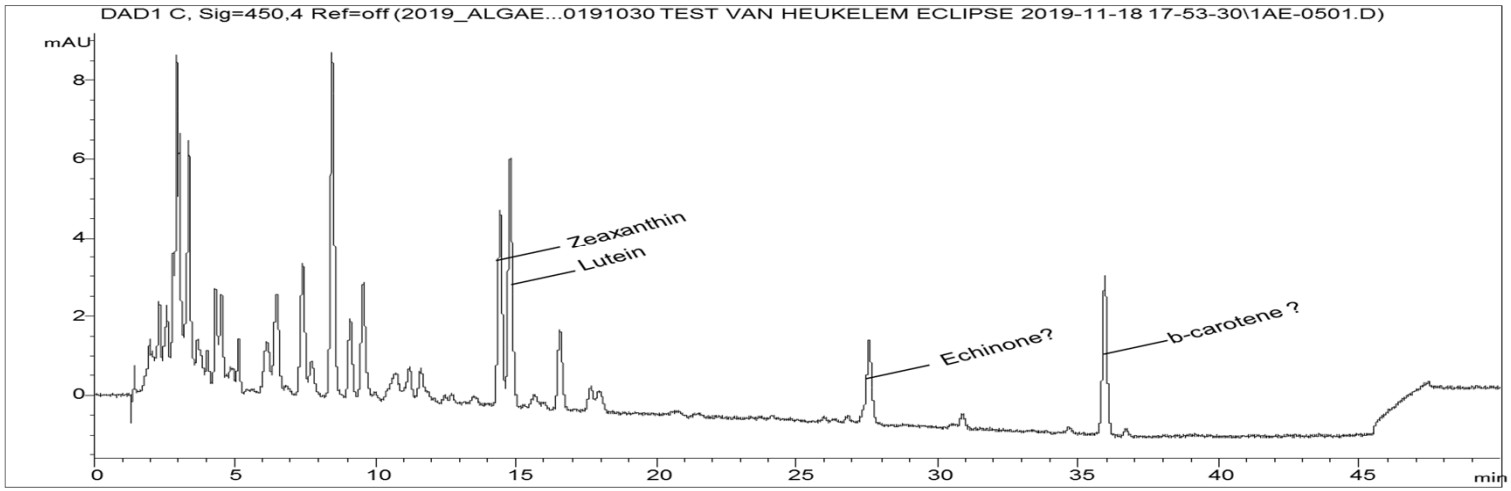
Hexane

Extraction of carotenoids and chloropfyll derivatives

Spirulina



MeOH



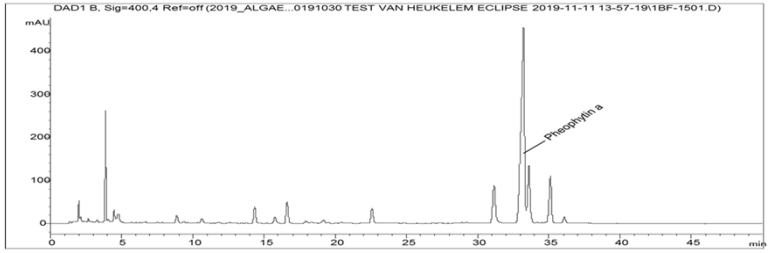
Hexane

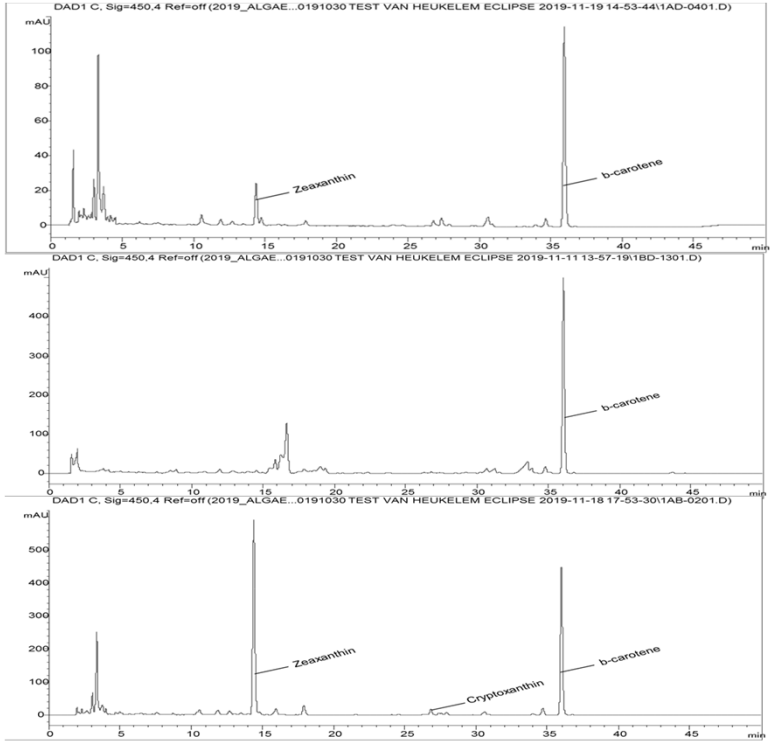
Analysis of algae oils

Algae Oils vs. alternative products

50 mg oil resuspended in
1. MeOH
2. Acetone
3. Ethyl acetate

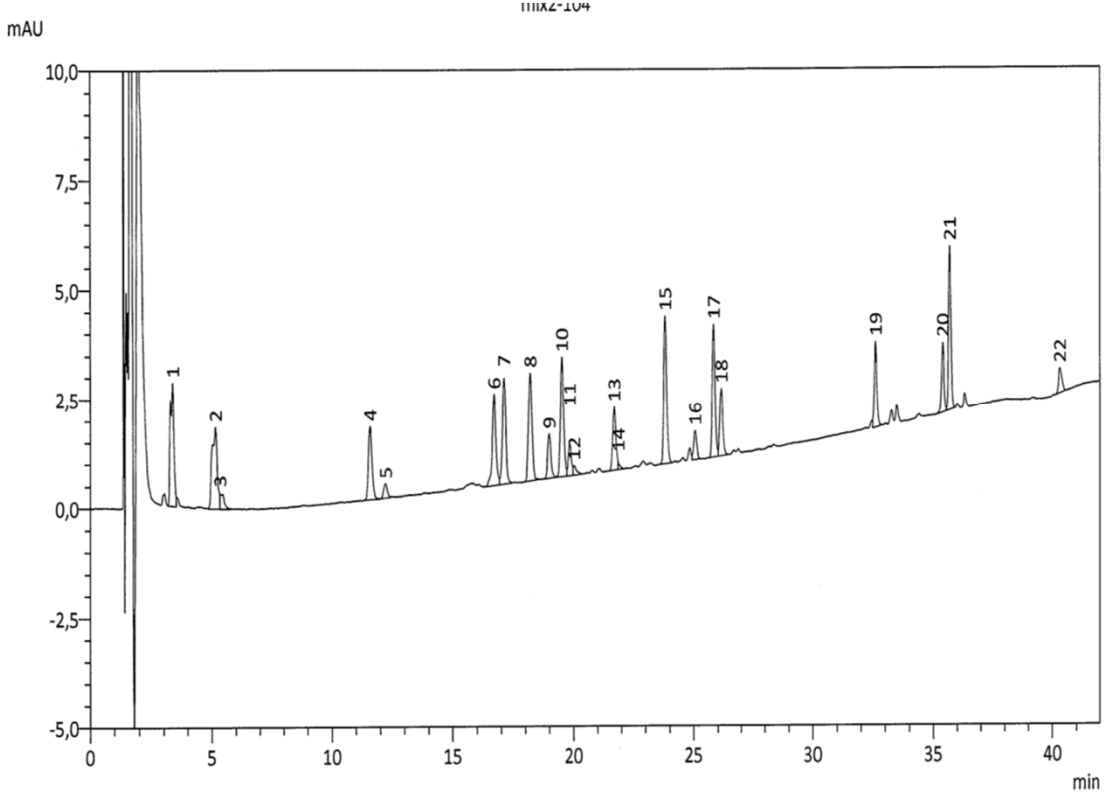
or
4. extrated with hexane method





Potential of the method I

DHI mix2-104

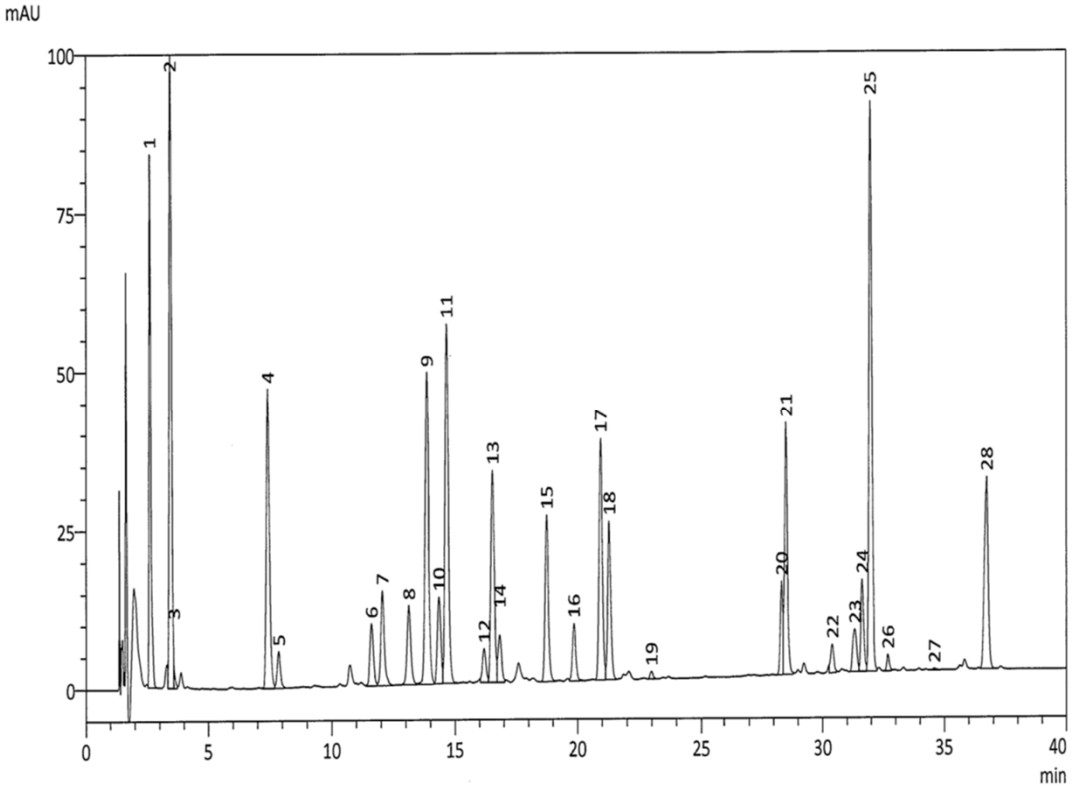


PDA Ch1 450nm

Peak#	Ret. Time	Name
1	3,37	Chlorophyll c3
2	5,15	Chlorophyll c2
3	5,33	Chlorophyllide a
4	11,57	Peridinin
5	12,20	Peridinin isomer
6	16,70	19'-but-fucoxanthin
7	17,11	Fucoxanthin
8	18,19	Neoxanthin
9	18,98	Prasinoxanthin
10	19,51	Violaxanthin
11	19,83	19'-hex-fucoxanthin
12	20,02	Astaxanthin
13	21,69	Diadinoxanthin
14	21,86	Dinoxanthin
15	23,81	Alloxanthin
16	25,05	Diatoxanthin
17	25,81	Zeaxanthin
18	26,13	Lutein
19	32,60	MV+DV chlorophyll b
20	35,39	DV chlorophyll a
21	35,69	Chlorophyll a
22	40,31	alpha+beta carotene

Potential of the method II

DHI mix-124



PDA Ch1 450nm

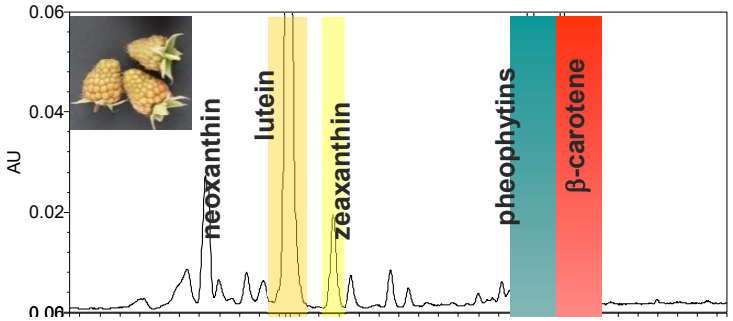
Peak#	Ret. Time	Name
1	2,61	Chlorophyll c3
2	3,44	Chlorophyll c2
3	3,57	Mg-DVP
4	7,41	Peridinin
5	7,84	Peridinin isomer
6	11,62	19'-but-fucoxanthin
7	12,05	Fucoxanthin
8	13,12	Neoxanthim
9	13,84	Prasinoxanthin
10	14,34	Violaxanthin
11	14,65	19'-hex-fucoxanthin
12	16,18	Diadinochrome
13	16,53	Diadinoxanthin
14	16,81	Dinoxanthin
15	18,73	Alloxanthin
16	19,85	Diatoxanthin
17	20,93	Zeaxanthin
18	21,27	Lutein
19	22,98	Gyroxanthin diester
20	28,31	DV Chlorophyll b
21	28,50	Chlorophyll b
22	30,39	Crocoxanthin
23	31,32	Chlorophyll c2-MGDG
24	31,62	DV Chlorophyll a
25	31,96	Chlorophyll a
26	32,68	Chlorophyll a epimer
27	34,58	Pheophytin a
28	36,71	Alpha+beta carotene

Isoprenoids in raspberry

unsaponified

saponified

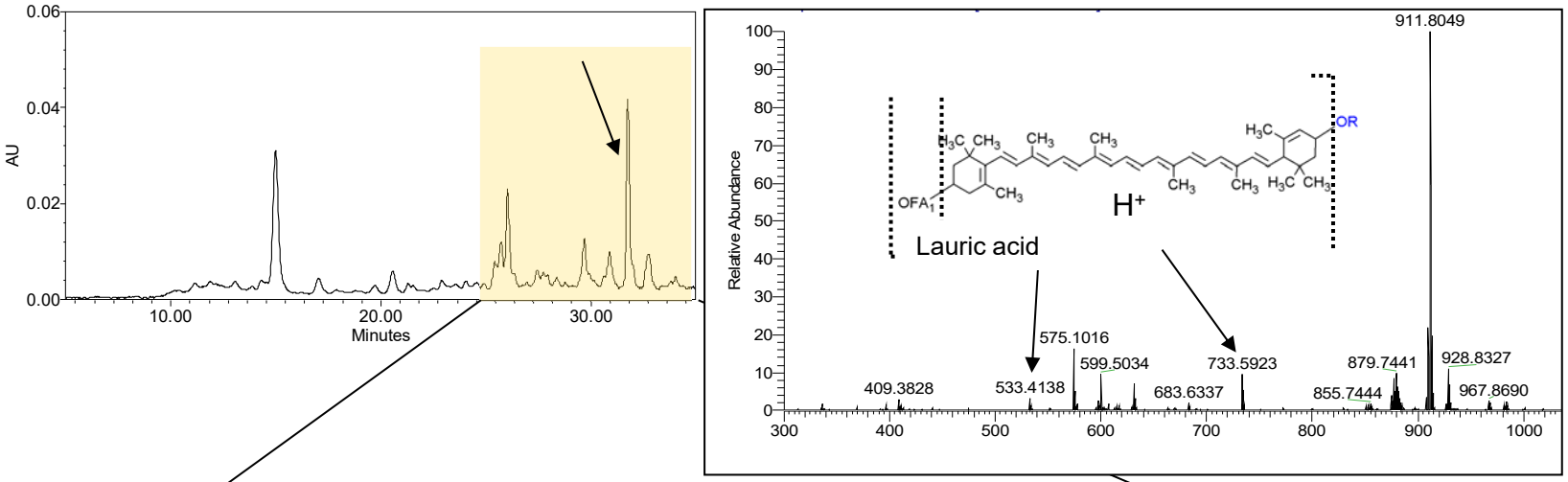
450 nm



Samples: Tulameen raspberry

Identification of lutein esters

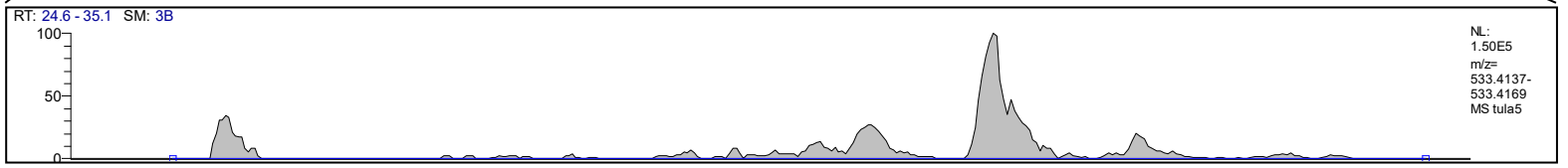
450 nm



FA$_1$=Lauric (C12:0), $[C_{52}H_{77}O_2]^+$, Theoretical *m/z*: 733.5929

Lutein backbone, $[C_{40}H_{53}]^+$: Theoretical *m/z*: 533.4153

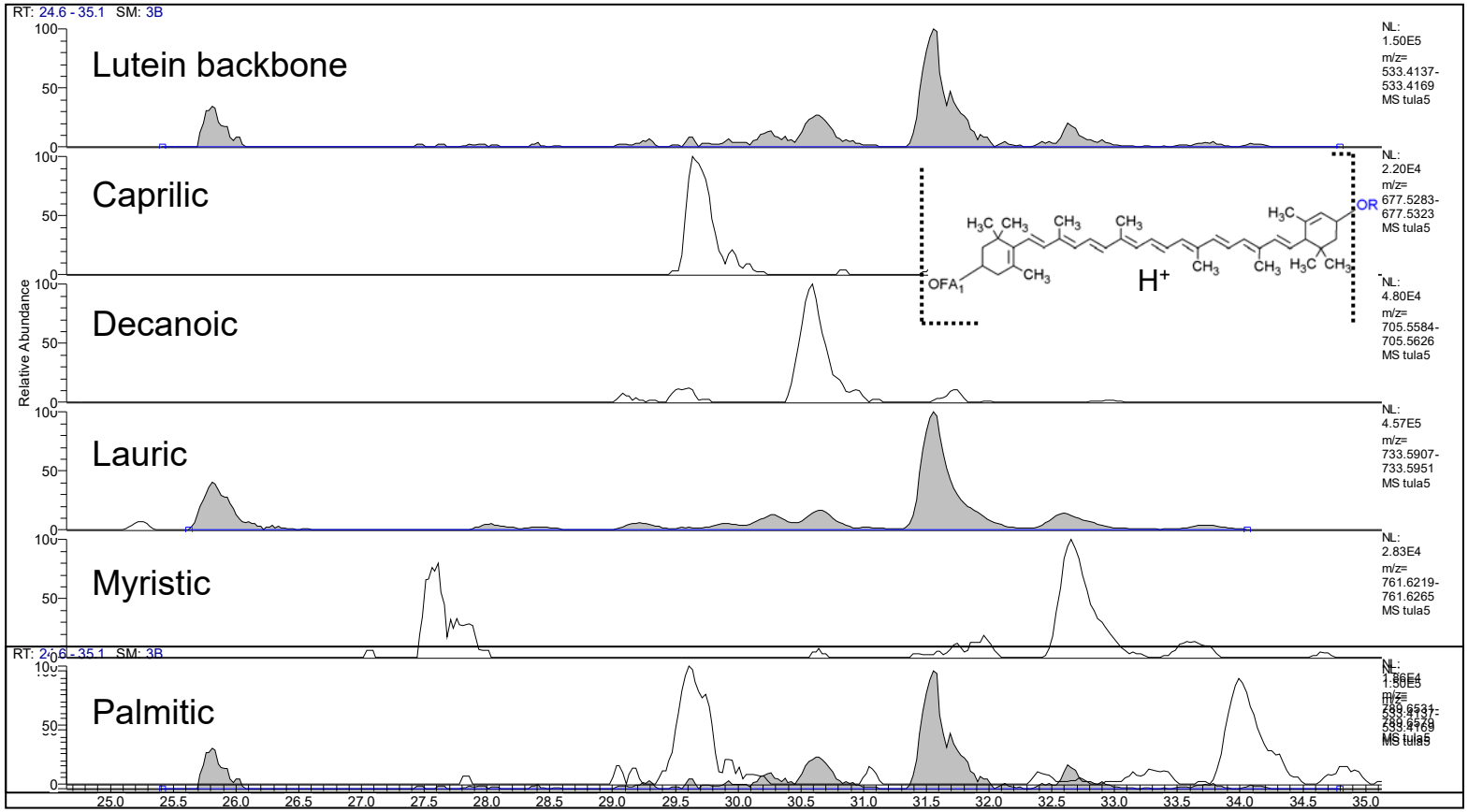
Extracted ion chromatogram
m/z 533.4153 (3 ppm)



Ripe Tulameen raspberry
RP C30 column, 3 μm (Fraser, 2000)
MeOH with 0.1 % FA post column, 5 μL/min
LTQ Orbitrap XL, APCI pos

m/z

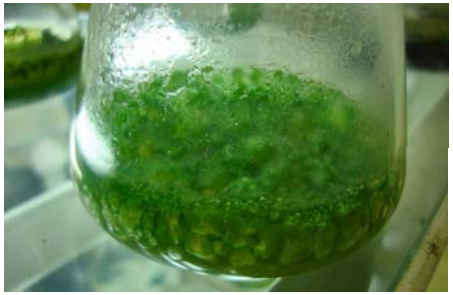
Identification of lutein esters



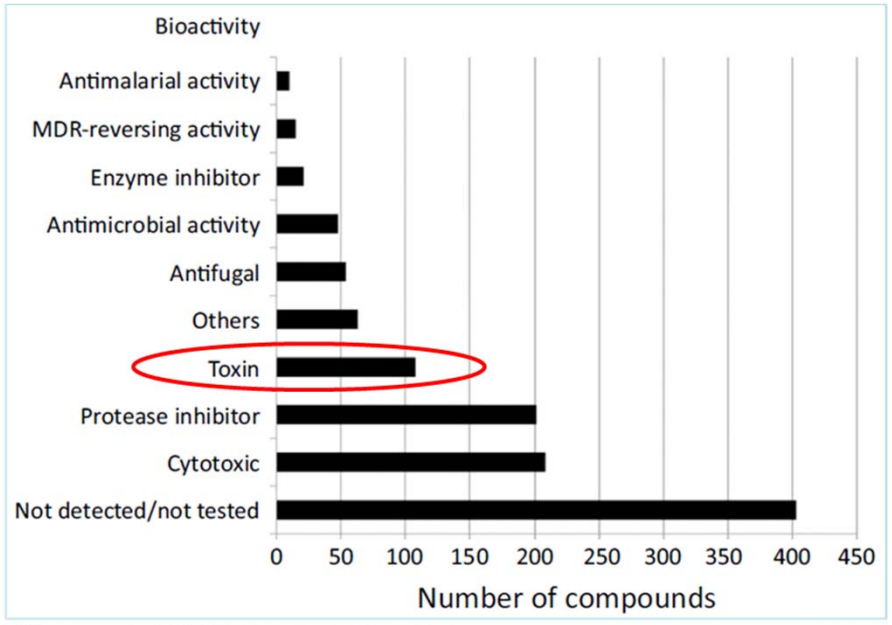
Ripe Tulameen raspberry
RP C30 column, 3 μm (Fraser, 2000)
MeOH with 0.1 % FA post column, 5 μL/min
LTQ Orbitrap XL, APCI pos

Metabolite analysis in microalgae – cyanobacteria

- microalgae have an extraordinary rich secondary metabolims
- many of these metabolites are also «bioactive»



Cyanobacteria («blue-green algae»), produce a wide panel of bioactive compounds.

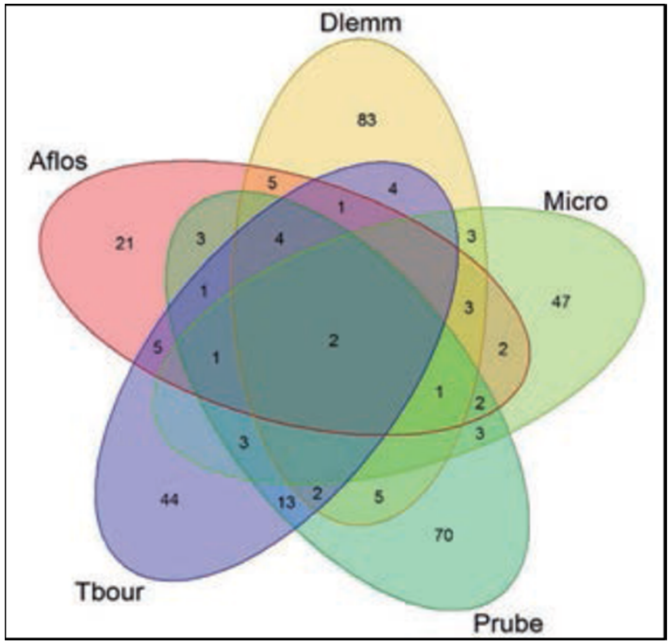


Dittmann et al, 2015. Trends in Microbiol.

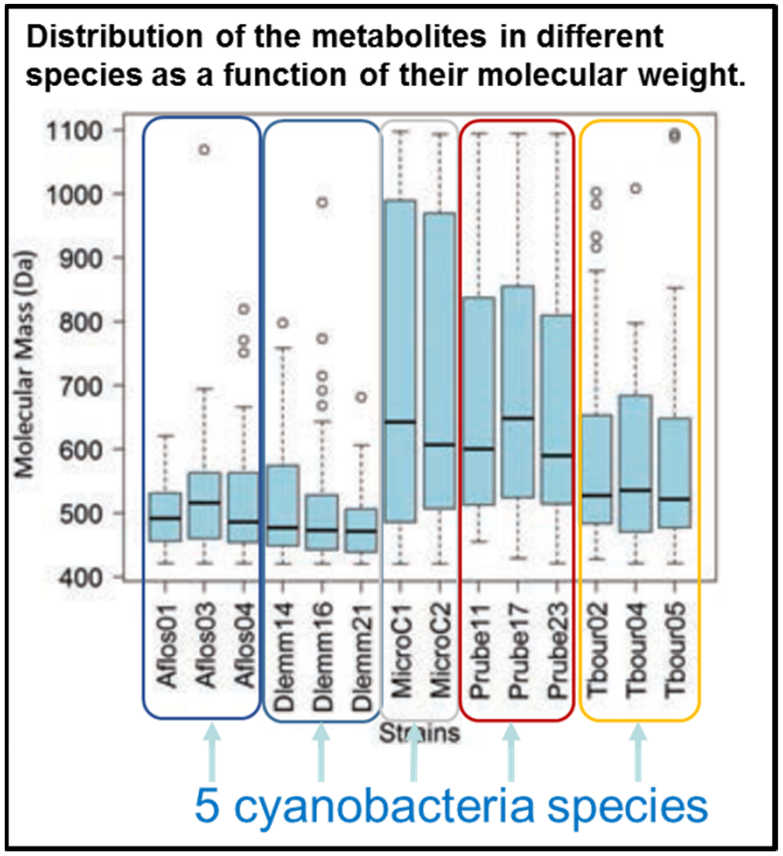
- more than 1100 secondary metabolites produced by cyanobacteria.
- from 39 genera.
- 731 active against diverse eukaryotic and prokaryotic cells, or inhibitors of various enzymes.

Comparative metabolomics in cultured cyanobacteria

- high diversity of secondary metabolisms in different species
- production of bioactive peptidic compounds (500-1000 Da) seems particularly developed in some species and almost absent in others



Venn representation of the metabolites diversity in 5 cyanobacteria species.



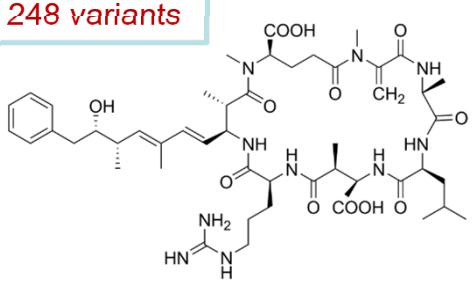
Toxins from cyanobacteria – classification

Concern in FRESHWATER

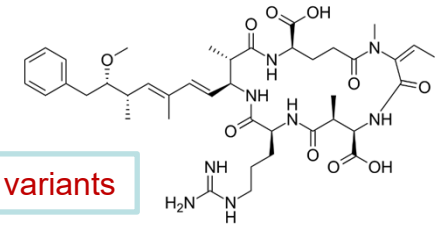
Non Ribosomal Peptides (NRP)

Microcystins (MW 900 - 1100 Da)

248 variants



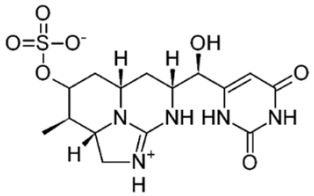
Nodularins (MW 800 – 900 Da)



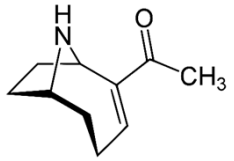
10 variants

Alkaloids and aminoacids

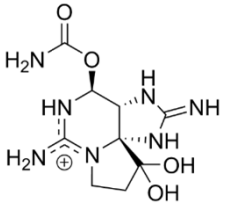
Cylindrospermopsins (MW 400 – 450 Da), 5 variants



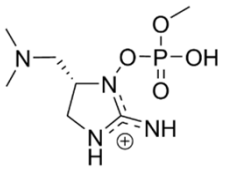
Anatoxins (MW 160 – 180 Da), 10 variants



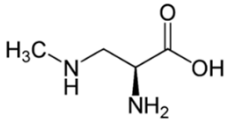
Saxitoxins (MW 350 – 500), 57 variants



Anatoxin-a(S)



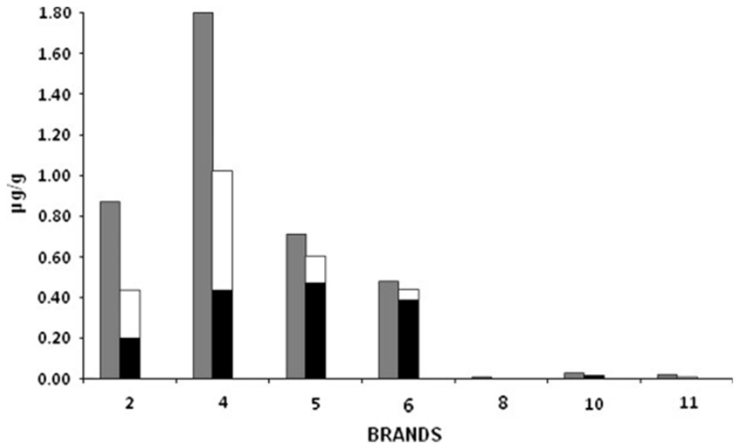
BMAA (β-methylaminoalanine)



*** Spoof and Catherine. 2017. Appendix 3. In: Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis. First Edition. Edited by Jussi Meriluoto, Lisa Spoof and Geoffrey A. Codd. John Wiley & Sons, Ltd.

«Toxinomics» for consumers' safety

Several studies have demonstrated the presence of microcystins in food supplements, due to the contamination of cultures with toxigenic cyanobacteria species.



Microcistins' content in food supplements for 11 brands measured with 2 techniques.
(Grey bars: ELISA; black/white bars: LC–MS/MS)

ELSEVIER

Food and Chemical Toxicology

Volume 50, Issue 12, December 2012, Pages 4493-4499

Contamination by *Microcystis* and microcystins of blue–green algae food supplements (BGAS) on the italian market and possible risk for the exposed population

Susanna Vichi, Paolo Lavorini, Enzo Funari, Simona Scardala, Emanuela Testai

toxins

MDPI

Article

Detection of Cyanotoxins in Algae Dietary Supplements

Audrey Roy-Lachapelle [1], Morgan Solliec [1], Maryse F. Bouchard [2] and Sébastien Sauvé [1,*]

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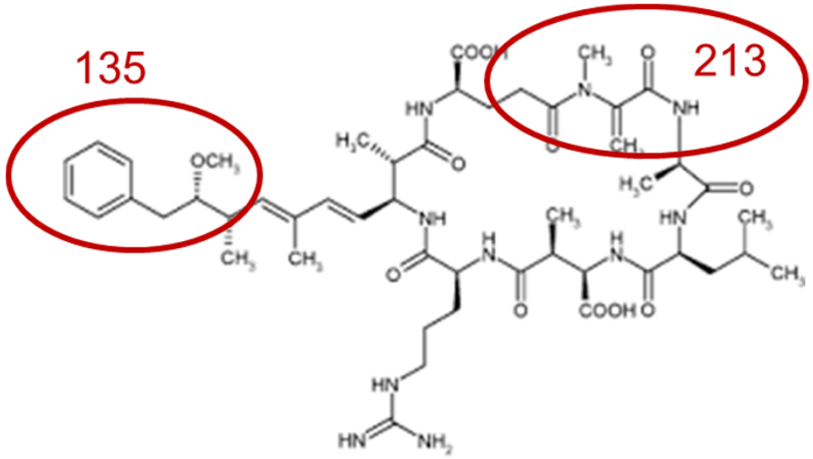
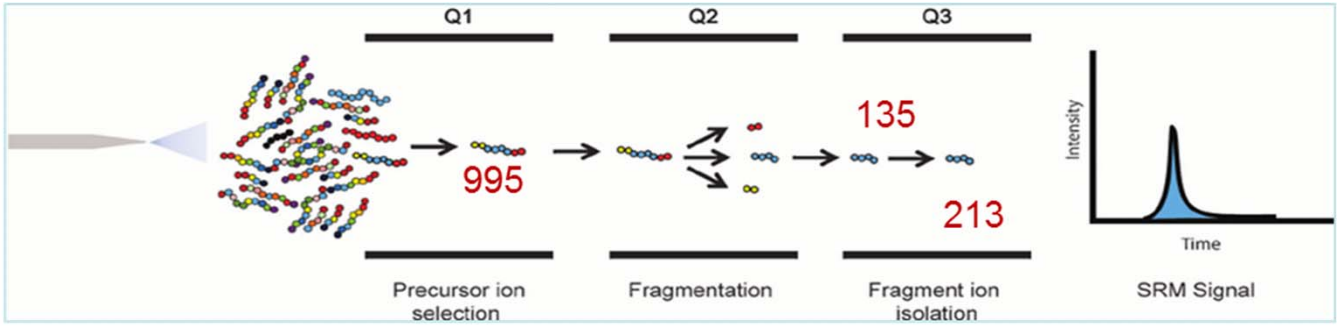
Academic Editor: Amparo Alfonso
Received: 6 December 2016; Accepted: 21 February 2017; Published: 25 February 2017

Target analysis of cyanotoxins

Identification and quantification of microcystins by target analysis (LC-MS/MS with Selected Reaction Monitoring)



135 and 213 are typical diagnostic fragments in microcystins



SRM analysis: high sensitivity and specificity.

Toxins' analysis in commercial products

Controlled closed cultivation

Summary Report:

Sample id	Analytes	value
Strain' s code	**MC**: RR, YR, HTyrR, LR, WR, LA, LY, LW, LF; **Demethylated MC**: *mono** and *bis** demethylated variants of the above parent MC; Nodularin; **Anatoxins:** ATX-a, HomoATX-a; Cylindrospermopsin; **PSP:** GTX1/4, C1/2, neoSTX, GTX5, STX, dcSTX, dc-neoSTX*, GTX2/3*, dcGTX2/3*	
A11		< LOD for all toxins
B16		< LOD for all toxins
C07		< LOD for all toxins
C08		< LOD for all toxins
C09		< LOD for all toxins
C10		< LOD for all toxins
C11		< LOD for all toxins
C12		< LOD for all toxins

*: some compounds were tentatively analyzed. No standards were available for confirmation.

Analysis were performed, after **extraction** from biomass and **purification with SPE**, with **LC-MS/MS** using a Waters Acquity UPLC coupled to a Sciex 4000Qtrap mass spectrometer. Peptidic compounds were analyzed with RP-C18 chromatography, while alkaloids were analyzed with HILIC.

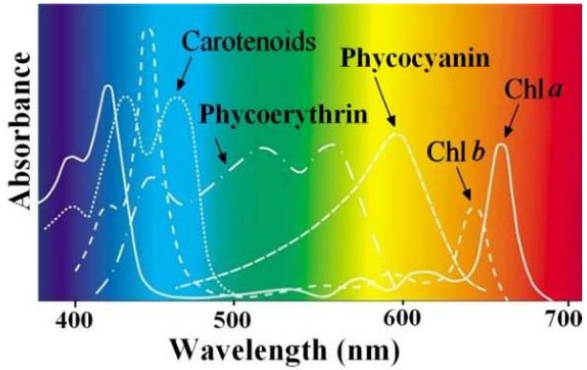
Limits of detection for each toxin:

analyte	LOD (ng/g dry biomass)
RR	*6.0*
dmRR	*3.5*
YR	*8.0*
dmYR	*8.0*
LR	*2.0*
HTyrR	*8.0*
dmHTyrR	*7.0*
dmLR	*2.0*
WR	*1.5*
LA	*12.0*
LY	*13.5*
LW	*22.5*
LF	*23.5*
Nodularin	*8.0*
ATX-a	*9.0*
HomoATX-a	*4.5*
Cylindrospermopsin	*1.0*
GTX1/4	*33.5*
C1/2	*22.5*
neoSTX	*67.5*
GTX5	*11.0*
STX	*22.5*
dcSTX	*45.0*

Pigments in algae

Pigments in microalgae strains

- reddish algae extract
- different strains change colour
    - cultures successfully established at FEM (HB)
        - **metagenomics**
        - *culture conditions for pigment synthesis*
        - *pigment isolation and structural elucidation*



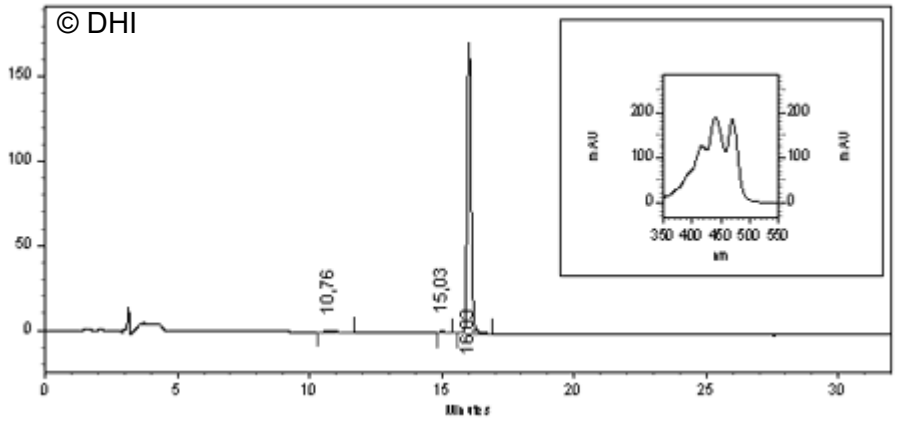
Microalgae cultures in the CSIRO Microalgae Collection laboratory.
http://www.scienceimage.csiro.au/image/2970

Application in aquatic ecology and RISE project

- Carotenoid and phenolic fingerprint of cultivated algae
  - Chlorella
  - Spirulina
  - others

MS-RISE AlgaeCeuticals

Biotechnological exploitation of algae for the production of cosmetics, food. Furthermore, the project will enhance knowledge exchange between academia, research centres and industry




K3

From cultivation to application