Project partners:

1. **Commissariat à l'Energie Atomique et aux énergies alternatives, CEA**
2. **The Scottish Association for Marine Science LBG, SAMS**
3. **A4F Algafuel SA, A4F**
4. **Karlsruher Institut fuer Technologie, KIT**
5. **Agencia Estatal Consejo Superior de Investigaciones Científicas, CSIC**
6. **Subitec GmbH, SUBITEC**
7. **PROTEUS, PROTEUS**
8. **MICROPHYT, MICROPHYT**
9. **SENSIENT Cosmetic Technologies, SENSIENT CT**

ABACUS

Algae for a biomass applied to the production of added value compounds

BBI 2016.R9 - Exploiting algae and other aquatic biomass for production of molecules for pharma, nutraceutic, food additives and cosmetic applications

Collaborative project

Start date of the project: 01/05/2017

Duration: 36 months

Deliverable D6.1

Physico-chemical characterization report of carotenoids produced in ABACUS

WP	6	Applicability
Task	6.1	Physico-chemical characterization of carotenoids

Dissemination level[1]	PU	**Due delivery date**	04/05/2018
Nature[2]	R	**Actual delivery date**	30/07/2018

Lead beneficiary	**MICROPHYT**
Contributing beneficiaries	MICROPHYT, CEA, A4F, Subitec, SENSIENT CT

[1] Dissemination level: **PU** = Public, **CO** = Confidential, only for members of the consortium (including the BBI), **CI** =Classified, information as referred to in Commission Decision 2001/844/EC.

[2] Nature of the deliverable: **R:** Document, report (excluding the periodic and final reports) , **DEM**: Demonstrator, pilot, prototype, plan designs, **DEC**: Websites, patents filing, press & media actions, videos, etc., **OTHER**: Software, technical diagram, etc.

WP 6 :	MICROPHYT	**Author**	Rémi Pradelles
	MICROPHYT	Approval by WP leader	Rémi Pradelles
	CEA	Approval by coordinator	J.F. Sassi

Document Version	**Date**	**Author**	**Comments**[3]
V0	09/02/2018	M. Pérez (CEA)	Creation
V1	09/05/2018	R. Pradelles (Microphyt)	Fist version
V2	16/05/2018	M. Pérez (CEA)	V1 revised
V3	26/06/2018	R. Pradelles (Microphyt)	Final version

[3] Creation, modification, final version for evaluation, revised version following evaluation, final

Deliverable abstract

This deliverable D6.1 sets the standards for the physico-chemical characterization of carotenoid extracts produced in ABACUS.

WP6, which began in M6, first focused its work on the definition of:

- parameters to be quantified
- analytical methods to be implemented according to the identified parameters and according to the different analytical techniques available within the consortium

Then, in order to ensure traceability of results and intercalibrations between partners, WP6 targeted the definition of:

- the arrangements for storing and transferring samples between the partners, in order to preserve their characteristics
- the procedures for centralizing & sharing the data obtained.

This preliminary work will set the basis for the technical implementation of the methods that will be applied to characterize the extracts produced in the frame of ABACUS. In collaboration with WP5, which recently produced the first extracts, the samples will be distributed to the partners involved in characterization.

Table of content

1 Introduction

Among the most valuable products targeted and developed within the framework of the ABACUS project are extracts derived from microalgae biomass containing significant carotenoid content.

These terpenoids are a large and very diverse group of molecules, with more than 300 structures known to date [1].

The characterization of extracts in qualitative and quantitative terms is essential in order to evaluate i) the influence of extraction processes on the profile obtained for these molecules, ii) the repeatability of the whole process developed (from the culture of microalgae to extraction) and thus iii) to guarantee a given profile in carotenoids.

The particularity of an extract rich in carotenoids derived from microalgae is the presence in the form of traces of minor carotenoids, added to the fact that carotenoids can be esterified and can exhibit different molecular conformations (isomers in particular).

This last point is of utmost importance because depending on the conformation of a carotenoid, its biological activity may be different or even opposite. In addition, cis/trans ratios are markers to differentiate carotenoids obtained from chemical synthesis from those obtained from natural biosynthesis by microalgae.

In this sense, with the aim of approaching applicability of these high value compounds in the target markets, and in order to secure their competitive advantage against similar chemically-synthesized molecules, it is crucial to have a reliable method to characterize the specificities of the carotenoids contained in the ABACUS extracts.

2 Experimental

In the ABACUS workplan, the tasks carried out within WP6 are highly conditioned by the production of the first extracts obtained from WP4 and WP5 workflow.

From its onset, WP6 has focused on three axes:

- The identification of the different carotenoids to be quantified and characterized in terms of isomers and the identification of associated methods.
- The definition of the storage and transfer conditions for the samples to be distributed among partners in order to ensure a good quality of the intercalibration between them.
- The development of a documentation system allowing a traceability and a centralization of the data generated by the analyses to come.

The two last items aforementioned are currently validated by the partners involved.

2.1 Carotenoids and associated analytical methods

WP6's first approach was:

- to list the carotenoids of interest in connection with all WPs and in particular WP1 and WP2 which defined the target molecules and associated species/strains;
- to determine those which require a characterization of their isomeric forms;
- to list the analytical techniques available among the various partners and to crosscheck their adequacy to the aforementioned purposes.

Following this work, two types of analytical protocols were selected. A first approach based on quantification without using a separative method via global quantification by spectrophotometry. This allows a faster quantification, and is applicable in routine process control. However, this type of protocol is non-selective and its relevance is questioned, especially for carotenoid mixtures or in the

presence of molecules absorbing at wavelengths close to the carotenoids of interest (for example: chlorophyll).

The second approach consists in the use of HPLC or HP-TLC, which allows characterizing and quantifying in a more precise way the extracts in terms of carotenoids. In the case of the use of a column type C30, the separation of the different isomers of the carotenoids becomes then possible. The disadvantage of this approach remains the duration of operation.

In fact, depending on the purpose (either quick-check or comprehensive profiling), the two aforementioned approaches are complementary (Table 1).

Table 1. Identification and quantification of carotenoids

			Processes of quantification and identification				
		Identification and quantification of isomerics forms	MICROPHYT	SAMS	CEA-Cadarache	A4F	CSIS
Carotenoids	Astaxanthin	**YES**	DMSO and/or Methanol extraction Desterification step with cholesterol esterase (from fungi)_Dilution in MetOH +BHT_HPLC DAD-C30 column-MetOH/MtBe/H20, + internal standard (béta-apocarotenal)	DMSO extraction / spectrophotometric . EtOH extraction and HPLC DAD analysis (C8 / C18) (Serive 2017)	Total extraction (DMSO, Acetone or Methanol). Quantification with HP-TLC.	HPLC. Acetone extraction, Vydac TP54, C18 RT,metanol 100% 1mL/min isocratic elution, UV-vis at 450 nm and 600 nm	HPLC-DAD-APCI-MS (C30 column)
	Zeaxanthin	**YES**					
	Fucoxanthin	**YES**					
	Lutein	**YES**					
	B-caroten	**YES**					
	Vauxerioxanthin	**YES**					
	a-carotene	NO					
	Alloxanthin	NO					
	antheraxanthin	NO					
	9Z-B-carotene	NO					
	Canthaxanthin	NO					
	echinenone	NO					
	lycopene	NO					
	violaxanthin	NO					
Molecules other than carotenoids but quantifiable by the same process and reflecting the quality of the extract	Chlorophyll a						
	Chlorophyll b						
	Chlorophyll c2						
	Chlorophyll c3						
	pheophorbide a						

NB: Chlorophylls and their derivatives are included Table 1 since they can be quantified through the same procedures applied for carotenoids. However, they will be specifically evaluated as potentially valuable side-products in the frame of task 6.3.

2.2 Storage and transfer conditions of samples

After having defined the parameters of interest and the methods to measure them, the work of WP6 consisted in identifying the good practices allowing the intercalibration between the partners, and thus define:

- Conditions for sample transfers
- Quantity of samples for analysis
- Identify potential subcontractants and costs associated for analyses that cannot be carried out within the Consortium

These good practices are being validated by the partners, thereby allowing editing a good management chart, whose first version is shown in Table 2.

Table 2. Samples storage and transfer conditions

	Sample quantity (g)	Sample storage and transfert
Sample of extract for carotenoids quantification and identification	5	- Protected from light - N2 saturation in headspace - BHT (0,1 %) - Max.Delivery duration : 48h - Storage : protected from light, T = -18°C

The objective of this work is to anticipate samples size and properties and to guarantee the quality and traceability of the results. Indeed, because of their chemical structure, carotenoid molecules are very sensitive to the various oxidation mechanisms. In particular, conditions such as elevation in temperature, rise in oxygen content or the presence of light can alter the carotenoid pool, both in quantitative and qualitative terms [2].

2.3 Data analysis and centralization

In order to share a centralized traceability among consortium members, WP5 & WP6 partners have worked on defining a spreadsheet template. This will allow comparing the results obtained between the partners, thereby aiming at a successful intercalibration.

The validation criterion, which was defined according to the principles of "ICH Guideline; Validation of analytical procedures: text and methodology", is the coefficient of variation within and between partners for all the targeted carotenoids. This parameter makes it possible to characterize reproducibility. For that, three laboratories must quantify the same samples (at least 5 different) and the coefficient of variation must remain strictly lower than 20 %.

The first version of the traceability spreadsheet is shown below in Table 3.

Table 3. Intercalibration and traceability management file

WP	6	Applicability
Task	6.1	Physico-chemical characterization of carotenoids
Strain		XXXX
Extraction process Reference		XXXX
Sample Reference		XXXX

	MICROPHYT				
Molecule	Mean Concentration in Product (%, m/m)	Standard deviation (%; m:m)	C.V (%)	Proportion of molecule in carotenoids fraction (%)	Method
Astaxanthin					
Zeaxanthin					
Fucoxanthin					
Lutein					
B-caroten					
a-carotene					
alloxanthin					
antheraxanthin					
9Z-B-carotene					
canthaxanthin					
echinenone					
lycopene					
vaucheriaxanthin					
violaxanthin					

The final version of this working document will be validated by all partners.

3 Conclusion

Tools and methodologies for the analysis of carotenoid profile and content from extracts produced by the ABACUS project have been defined. The various technical coordination, traceability and data centralization systems will be practically tested over the coming months. Indeed, following the work of WP1 (definition of target substances) to 5 (development of fractionation protocols), the first extracts were produced. The first characterizations will focus on microalgae extracts containing astaxanthin, zeaxanthin, beta-carotene or vaucheriaxanthin.

The main bottleneck to overcome is to find a suitable source for standards of carotenoid isomers which will used as reference material in method development and validation. Some have already been identified. For those whose supply appears to be problematic, custom synthesis based on the application of the photoisomerization process, may be implemented [3].

4 References

[1] C. C. C. &. S. C. Galasso, «Carotenoids from Marine Organisms: Biological Functions and Industrial Applications,» *Antioxidants, 6,* p. 96, 2017.

[2] F. Ladislav, V. Pacáková, K. Stulík et K. Volka, «Reliability of Carotenoid Analyses: A Review,» *urrent Analytical Chemistry. 1,* 2005.

[3] R. Aman, J. Biehl, R. Carle, J. Conrad, U. Beifuss et A. Schieber, «Application of HPLC Coupled with DAD, APCI-MS and NMR to the Analysis of Lutein and Zeaxanthin Stereoisomers in Thermally Processed Vegetables,» *Food Chemistry. 92,* pp. 753-763, 2005.