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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

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Deliverable D6.2

Physico-chemical characterization report of terpenes (short chains) produced in ABACUS

WP	6	Applicability
Task	6.2	Physico-chemical characterization of terpenes

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Deliverable abstract

This deliverable D6.2 gives the different possible techniques available for the physico-chemical characterization of terpenes (short chains) produced in ABACUS.

Table of content

Abbreviations

DHS Dynamic Headspace Extraction
FID Flame ionization
FMAH Focused Microwave-Assisted Hydrodistillation
GC Gas chromatography
GPPS Geranyl diphosphate synthase
HS Headspace Extraction
HPLC High-performance liquid chromatography
HD Hydrodistillation
LC Liquid chromatography
LMW Low molecular weight
MS Mass spectrometry
SE Solvent extraction
SFE Supercritical Fluid Extraction
SHS Static Headspace
SPME Solid-phase microextraction
VOC Volatile organic compound

1 Introduction

Terpenes are the largest group of natural products with more than 22,000 individual compounds known at present. The fundamental building block of terpenes is the isoprene unit (2-methyl-1,3-butadiene). According to their chain length, terpenes are classified into hemiterpenes (C5), monoterpenes, (C10), sesquiterpenes (C15), diterpenes (C20), sesterpenes (C25), triterpenes (C30), tetraterpenes (C40, carotenoids) and polyterpenes ((C5)n with n >8). The term terpene or terpenoid (also called isoprenoid), include both hydrocarbon terpenes and their oxygen containing derivatives where methyl groups have been moved or removed, or oxygen atoms added [1] [2].

Terpenes present several roles in nature, such as plant photoprotection and light harvesting, defense against herbivores and fungi, attraction for pollinators or, in mammals, contribution to cell membrane stabilization metabolic pathways or in the regulation of certain enzymatic reactions.

Regarding their potential commercial applications, these compounds are gaining much attention as they could not only prevent but treat several diseases (aromatherapy), could act as natural antimicrobial and insecticide agents in agriculture or even constitute the basis for the synthesis of biofuels, solvents and high value compounds [3] [4].

Light mono- and sesqui-terpenes commonly occur in the essential oils that are responsible for plant fragrances (Figure 1). Therefore, these compounds have been traditionally used for centuries to elaborate fragrances and flavors. Monoterpenes, that are formed by two isoprene units, are derived from geranyl diphosphate (GPP) and can be classified into cyclic and acyclic ones. Detailed information on monoterpene biosynthesis can be found in Figure 2.

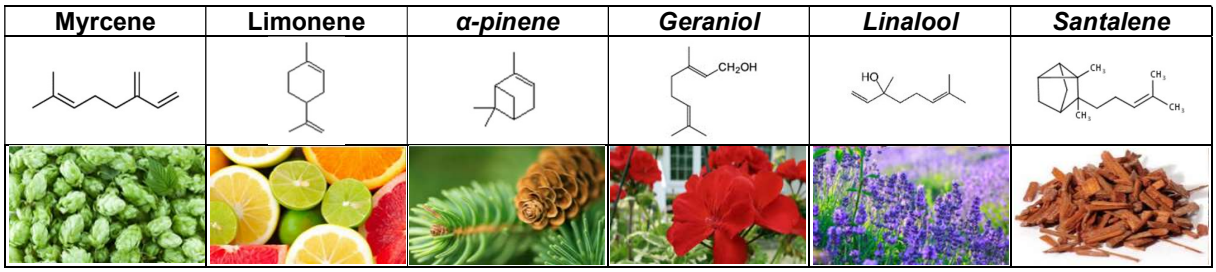

Figure 1. Examples of terpenes and corresponding natural plant sources. Photos from left to right: myrtle, citrus, pine tree, geranium, lavande, sandal wood

Within this report, we will focus only on the characterization of the volatile, low molecular weight terpenes that have been identified within ABACUS Project as target innovative compounds for fragrances and cosmetics applications. On the main approach, we will focus on the production and quantification of some common terpenes such as those included in Figure 1 by means of genetically modified cyanobacteria. Furthermore, other types of VOCs with potential application in fragrances and produced by microalgae metabolism (e.g. volatile aldehydes) might be considered too.

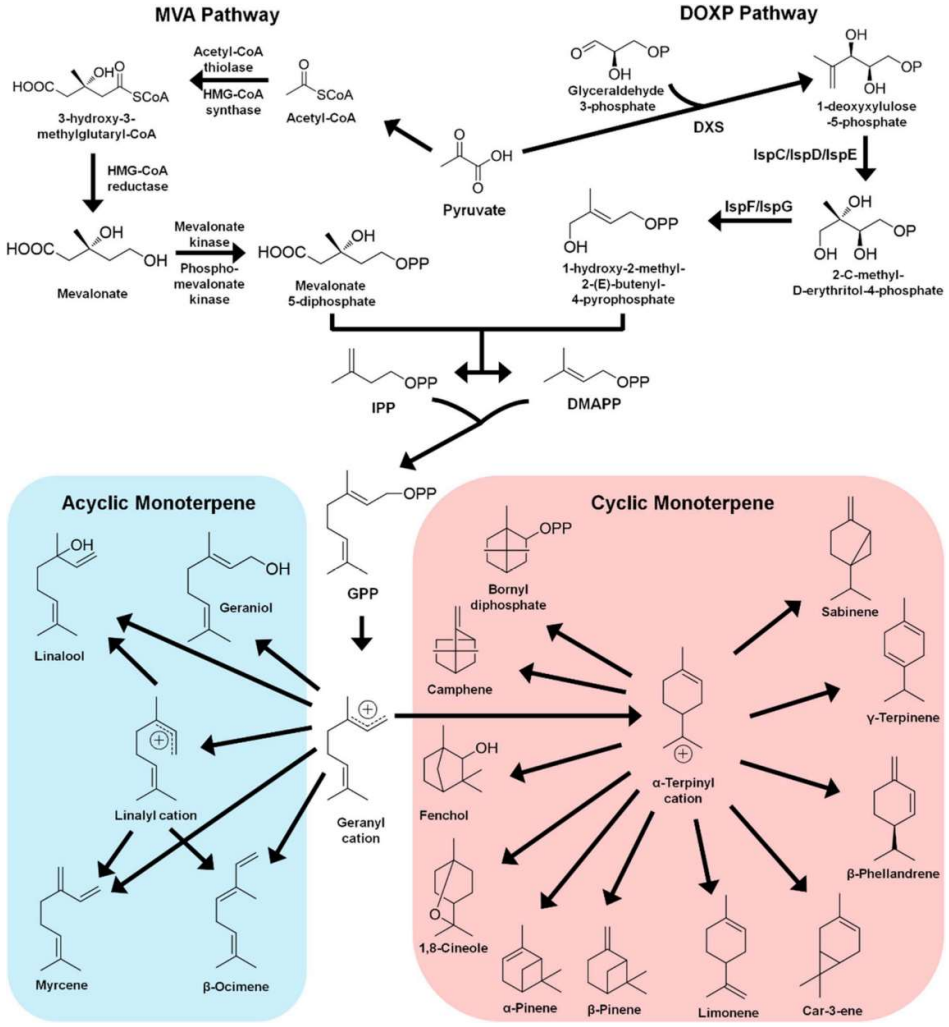

Figure 2. Monoterpene biosynthetic pathways and product diversity [5].

2 Methods for Terpene Quantification

Terpene extraction is typically achieved by solvent extraction or steam distillation. In addition, analysis of terpenes conjugated to fatty acids or other acyl derivatives including ester bonds require a prior saponification step to be released. Then, quantification is most often performed by a chromatographic method. In this section, the most commonly used methods for terpene extraction and quantification are described.

2.1 Extraction methods

Solvent extraction (SE)

Solvent-based extraction is one of the most common methods for terpenes. It will also be considered as a reference method within ABACUS project (reference protocols can be found in public deliverable D2.1). In the case of volatile organic compounds (VOCs) extraction, typically performed with plant or algal biomasses, a solid-liquid continuous extraction system is applied, where a solid matrix is washed with the solvent in a Sohxlet device. The type of solvent, the solvent/sample ratio, granulometry, time of contact and temperature of extraction are the variables that condition overall extraction efficiency and that need to be tuned on a case by case basis [6]. Therefore, an optimization

process is required where most suitable conditions are determined for an enhanced product recovery. The choice for the appropriate solvent will depend on the compound to extract and the characteristics of the biomass, with hexane, ethyl ether and dichloromethane being the most commonly used.

Solvent extraction can be also used for in vivo monitoring of volatile compounds in microalgae by placing a layer of dodecane over the surface of the growth medium for a certain period of time. Time of exposure to dodecane must be optimized in order to avoid toxic effects on the cultures [7].

Although this is a well-known technique, it has many disadvantages such as the toxicity and environmental pollution related to some solvents, the low concentration of the extracted compounds, the loss of volatile compounds during different extraction steps or even their degradation.

Hydrodistillation (HD)

Vapor distillation or hydrodistillation are traditional methods for VOCs extraction, usually performed in Clevenger or Dean-Stark devices, where heated water is used to achieve volatilization of the volatiles that are dragged by the steam. Since they are simple and direct techniques, they are still largely applied for aromas and fragrances characterization either alone or combined with other sample-preparation steps. Nevertheless, they might also present some problems related to loss of volatile products, low extraction efficiency or partial degradation of these compounds due to high temperatures. Quantification can be achieved by a chromatographic method [8] [9].

Focused Microwave-Assisted Hydrodistillation (FMAH)

This technique, first described by Ganzler et al. in 1986 [10], has recently gained attention for the extraction of essential oils [8]. The device consist of a Clevenger or Dean-Stark apparatus coupled with a microwave oven equipped with a power modulator and a device for heat capture. In this case, volatilization of the compounds in a sample mixed with water is achieved by the microwave power in a faster way than traditional hydrodistillation. Still some optimizations need to be done in terms of irradiation power and extraction time in order to obtain optimal yields, but in principle this technique offers a lower operation cost than the traditional one.

Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE) was introduced by Zosel in the 1960s and can be performed both at small or industrial scale. It relies on an extraction achieved by a fluid kept above its critical pressure and temperature. At this state, supercritical fluids show high diffusivities and low viscosities, which provide good mass transfer properties, and makes the extraction process faster and more effective [11]. Pumped into an extraction vessel where the sample biomass is placed, the supercritical fluid passes through the sample matrix, solubilizes the compounds of interest (the VOCs in this case) and then takes them away to a device where they are collected and further analyzed by an analytical technique (e.g. chromatography).

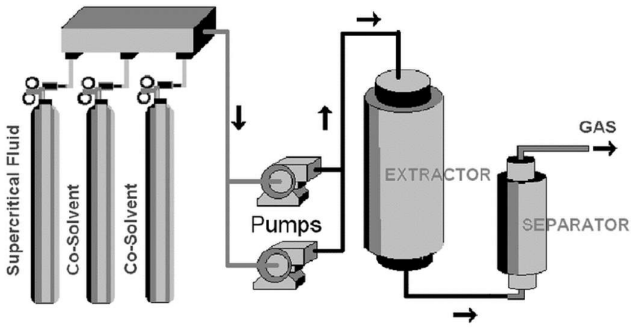

Figure 3. Schematic diagram of a supercritical fluid batch extraction [12].

In addition, SFE-based techniques can be considered green extraction techniques if appropriate conditions are selected. Nowadays, supercritical fluid extraction (SFE) is a widely spread technique

based on the use of high pressures for the extraction of natural bioactive compounds and those target compounds identified within ABACUS project.

The most common supercritical fluid used is CO_2, as it has a low pressure and a low temperature critical point (71 atm and 31°C, respectively). Moreover, it is non-oxidizing, non-toxic, non-flammable, food-compliant, easily available and it is able to solubilize, among many other compounds, mono and sesquiterpenes, which are target compounds in ABACUS.

Headspace Extraction (HS)

Headspace extraction is a solvent-free technique based on directly collecting volatiles produced as vapors by the algae and transferring them to a gas stream that is further analyzed in gas capillary columns. Main parameters to optimize in HS include temperature, gas-liquid equilibrium and extraction periods. Three different sampling techniques are possible within HS: static headspace (SHS), dynamic headspace (DHS) and solid phase microextraction (SPME) (see Figure 4).

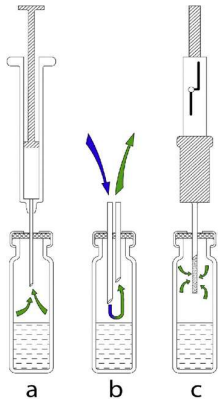

Figure 4. Headspace sampling techniques: a) Static headspace, b) Dynamic Headspace Extraction and c) Solid-phase microextraction [13].

- Static Headspace (SHS). In this method, the biomass is placed in a closed vial and a controlled temperature is applied. The released VOCs are then captured from the headspace of the vial with a syringe and taken into a GC-MS for analysis. Since it is a very simple method, it can be applied to detect compounds with low or medium partition coefficients, such as aqueous solutions containing volatile hydrocarbons, terpenes, lower mercaptans, sulfides, disulfides, carbonyl compounds and ethers, as well as dissolved gases. However, since there is no concentration step considered, this method might be limited when it comes to quantitative analysis due to a low sensitivity. Nevertheless, SHS can be very suitable for fragrances and aroma analysis when the technique and the devices have appropriate detection limits.
- Dynamic Headspace Extraction (DHS). A large number of studies present DHS as a suitable method for chemical characterization of fragrances and aromas. It offers a higher sensitivity than SHS but also requires more complex instrumentation and procedures. This technique consist of separating the volatiles from a solid or liquid biomass sample by the continuous flow of an inert gas (tipically Helium) that carries out the VOCs through an adsorbent material where they get trapped and concentrate. After this process, a thermal desorption step is applied and the compounds are analyzed by a chromatographic method.
- Solid-phase microextraction (SPME). This method performs extraction and concentration in a single step, providing high analytic performance with less sample manipulation compared to other methods. The extraction is based on an adsorption/desorption of VOCs on an inert polymer-lined fiber placed at the headspace of a hermetically closed flask. The fiber is exposed for a certain time to the released VOCs, and later on removed and inserted into the injector of the GC, where volatiles are thermally desorbed and analyzed. Time and adsorption temperature are the main parameters to optimize, and the use of an internal standard is recommended.

For example, in the method proposed by Wu et al. (2016) [14], the volatile terpene compounds from the headspace of several cultures of fungi were analyzed by extracting VOCs with a preconditioned SPME syringe consisting of 50/30 divinylbenzene/carboxen on polydimethylsiloxane on a Stable Flex fiber followed by GC-MS. The SPME fiber was exposed into the headspace of each culture flask for an hour to saturate with the volatile terpene compounds produced by the various TPS-expressing strains. The syringe was then inserted into the injection port of a Varian 3800 gas chromatograph containing a 30mx0.25mm i.d DB waxer capillary column with a film thickness 0.25µm. The column temperature was programmed as follows: 60°C for 4 min, increasing to 120°C at 10°C/ min and holding for 5 min, then increasing to 220°C at 20°C/min and holding for 2 min, then increasing to 250°C at 50°C/min and holding for 4 min. The carrier gas was ultra-high purity helium at a constant flow rate of 1 mL/min, and the initial column head pressure was 50Kpa. A two minute injection time was used to desorb the terpene compounds from the sampling fiber into an injection port (splitless mode, injection temperature—220°C) of the chromatograph coupled with a Saturn 2000 ion trap mass spectrometer. The MSD parameters were EI at 70eV, mass range was 30–500 Da, and the scan speed was 2 scans/sec.

2.2 Quantification methods

The most common technique for VOCs characterization after extraction is to couple a strong separation technique, such as gas chromatography (GC) or liquid chromatography (LC) with a sensitive detection method, like mass spectrometry (MS) or flame ionization (FID) [15].

GC involves a mobile phase consisting of an inert carrier gas, (typically helium), and a stationary phase placed inside the GC column, with different affinities for the volatiles. As they pass through the column, the VOCs interact with the stationary phase and each volatile will elute at a different retention time, according to the degrees of partitioning of the VOC between the mobile and the stationary phase. The temperature of the CG column can be increased in a ramp mode in order to separate compounds with close boiling points. Output signal of the detector is converted into a chart (gas chromatogram) and allows detecting concentrations down to the order of parts per trillion (ppt).

With an MS detector, high energy electrons are applied over the sample colliding with the atoms of the volatile compounds and generating positively charged particles. These charged particles are often separated magnetically and detected based on their position and relative abundance, yielding a mass spectrum (mass/charge). Other detectors can employ different physicochemical principles such as thermal conductivity (TCD), flame ionization (FID) or photoionization (PID) among many others.

In fact, the GC-MS technique is able to detect a wide range of compounds, either polar or non-polar. During the analysis, mass spectra of extracted compounds is compared to reference data from mass spectral libraries in literature (e.g. NIST) in terms of retention index (RI) relative to an internal standard mixture of n-alkanes [7] [16] [17].

The use of a couple high performance liquid chromatography and MS (HPLC-MS) has also many applications, especially for compounds more difficult to volatize. Although less common for VOCs analysis, there are some studies applying this technique [18].

Nuclear magnetic resonance (NMR) is a sophisticated spectroscopic method for analyzing a wide variety of materials and it is one of the main techniques used to obtain physical, chemical, electronic, and structural information about a molecule. One-dimensional ^{1}H NMR and ^{13}C NMR (DEPT 45, DEPT 90 and DEPT 135) and two-dimensional homo- and hetero-nuclear ^{1}H-^{1}H (COSY), ^{1}H-^{13}C (HMQC) and ^{13}C-^{13}C (HMBC) techniques have been applied to the identification of unknown macroalgae metabolites [19] [20] [21].

3 Methods recommended for ABACUS samples

For in-vivo and in-vitro monitoring of terpenes in ABACUS trials, we will apply solvent extraction/collection followed by GC-MS identification and quantification.

Furthermore, a simpler UV-VIS spectrophotometry procedure has been identified for the determination of overall terpene content. Complete procedure can be found in Annex.

For the development of online VOC monitoring devices, micro-chip headspace methods combined with GC-MS are being explored so far in WP2 .

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5 Annex: Determination of total terpenes

This simple protocol will be used in ABACUS to quantify globally the terpene content in algal biomass/algal extracts [22].

Materials

- Dried biomass
- 1.5 mL micro-centrifuge tube
- 2 mm glass beads
- 2 mL HPLC vials with Teflon lined screw cap
- Bead mill
- Hot Block
- Centrifuge
- Vortex
- Spectrophotometer
- Quartz cuvettes
- Pasteur pipettes
- 15 mL centrifige tubes
- Distilled water
- Heptane
- Squalene

Safety Issues

- Standard lab safety should be followed.
- Exercise care when handling chemicals.
- Care should be taken when handling hot samples.

Procedure

To note beforehand:

- There are possible interferences between heptane and plastics.
    - Any step involving heptane should use glassware if possible
        - In a worst-case scenario, use pipette tips only once.
- This method does not currently work for cyanobacteria or *Porphyridium*.
    - The aqueous/solvent mixture froths immediately upon vortexing. This suggests the presence of saponins/surfactants.
    - These micro-organisms also have phycobiliproteins present
        - A method development will attempt an initial extraction of these compounds with a phosphate buffer (see method 2.19), before being subjected to the following procedure.

Procedure:

- Weigh 2±0.25 mg dried biomass
- Transfer to a 1.5 mL micro-centrifuge tube
- Add 3-5 glass beads
- Add 1 ml of dH_2O, resuspend by pipetting.
- Bead mill for 6 minutes at 30 Hz.
- Transfer aqueous solution to a 2 mL HPLC vial
- To this, add 1 mL of heptane.
    - This should separate into an upper solvent, and lower aqueous phase.
- Replace, and tightly fasten the teflon lined cap.
- Vortex, at high speed, for 1 minute.
- Place in a hot block at 100°C for 1 hour.
- Remove, and allow cooling to room temperature.
- Vortex for 1 minute
- Centrifuge for 10 minutes at 1000 rpm
    - If necessary, place 2 mL HPLC vial inside a 15 mL centrifuge tube.
- Transfer the upper solvent phase to a quartz cuvette using a glass Pasteur pipette
- Read upper solvent phase at 220nm and 750nm.
    - 220nm for terpene quantification
    - 750nm for quality control (*i.e.* ensuring that there are no debris)
- A blank is composed of heptane only
    - Measure absorbance of the blank several times before analyzing samples to ensure stability

Standard Curve:

- Create a 5 mg/mL squalene stock solution in heptane
    - Avoid repeat use of plastic pipette tips
    - Avoid plastic is possible, use glass Pasteur pipettes and volumetric flasks if possible
- Generate an appropriate dilution series of 0-5 mg/mL squalene in heptane
- Read at 220nm
- This creates a logarithmic standard curve.

Please note, the following representative (backbone) terpenes from different terpene classes may also be considered as a standard.

- Monoterpenes (C10) = geraniol
- Sesquiterpenes (C15) = farnesene
- Diterpenes (C20) = phytane
- Sesterterpenes (C25) = Pentamethylicosane or haslene
- Triterpenes (C30) = squalene
- Tetraterpenes (C40) = Phytoene or β-carotene