Open-BIO

Opening bio-based markets via standards, labelling and procurement

Work package 3

Biomass Content

Deliverable N° 3.7

Direct bio-content automation

Public report

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1 Summary

The Open Bio project (www.open-bio.eu) intends to increase the knowledge of standard and certification system for bio-based products. In the frame of test methods used for biobased content measurements, official methodology based on the determination of both ^{14}C determination and elemental analyses quantification (A.C.D.V. method) has been elaborated.
Other approaches have been investigated in the aim to obtain new complementary methods. Stable isotope determination which is a relevant method in the natural authenticity assessment for regular controls could be an alternative method and is cheaper and faster as the ^{14}C method.

This deliverable presents the different specifications of direct automation based on isotopic methods, analytical combinations, precision and upcoming improvement in this field. Isotopic analysis started after the Second World War with the development of Isotope Ratio Mass Spectrometer (IRMS). This device is able to determine the organic isotopic ratios in gas. In order to take into account various forms (solids, liquids) automatic elemental analysers (EA) are connected to isotope ratio mass spectrometer (EA-IRMS) for bulk sample materiel usually called Bulk Stable Isotope Analysis (BSIA). The methodology is easy to operate, carries out analyses in few minutes, has a relative low cost and provides elemental amount of the target elements and multi isotopic determinations. It is extremely appropriate for regular authenticity control of natural product and is well used in this scheme. Usually isotopic instruments are able to give the isotopic values of all organic elements contained in the samples.

Isotope ratio mass spectrometer can also be connected to separate devices prior to perform the isotopic determination for Compound Specific Isotope Analysis (CSIA). In this approach, isotopic ratios of individual components are determined in a mixture sample. Two different processes are available depending on the molecules investigated: Gas chromatography (GC) or liquid chromatography (LC) and thus 2 types of implements:

- Gas chromatography- combustion/pyrolysis –Isotope ratio mass spectrometer (GC-C/P-IRMS)
- Liquid chromatography- chemical oxidation- Isotope ratio mass spectrometer (LC-co-IRMS)

These implements can treat a mixture sample within few hours and are able to take into account sequences of components.
Isotopic determinations are expressed in delta notation and correlated to an international reference.

$$\delta(^{13}C/^{12}C) = \left[\frac{R(^{13}C/^{12}C)sample}{R(^{13}C/^{12}C)standard} - 1\right] * 1000$$

International standards are supplied by I.A.E.A. (International Atomic Energy Agency) and other organisms to guarantee the accuracy of the isotopic values obtained.
The analytical precision of multi-isotopic approaches carried out on modern equipment for BSIA or CSIA is given with high quality and enough to distinguish difference origins of targeted compounds.

The instrumental development of Position-Specific Isotope Analysis (PSIA) methodology for the determination of intramolecular carbon isotope measurements and Isotope Ratio Infrared Spectroscopy (IRIS) approaches offers new possibilities to bring complementary isotopic data. All of the evolutions of the direct automated methods permit to gain in accuracy, in performances and increase the field of investigations.

However, even if the stable isotope determination is a fully automated approach, the determination of “Direct bio-content automation” using stable isotope processes cannot be undertaken without substantial complements needed. It becomes a tool for a well-controlled process or at most a single step or organization in the value chain.

2 Introduction

Resource supply and environmental aspects are considered of increasing importance to industrial production. Materials and products based on renewable resources can contribute to both economically and ecologically efficient solutions. Therefore, it may be of interest to be able to determine and communicate the 'content of renewable resources' of a given material or product.

Element carbon, C, has an isotope, ^{14}C, which allows for a clear distinction between carbon based substances in present living organisms and carbon based substances from fossil sources. The ^{14}C present in chemicals originates from recent atmospheric CO_2. Due to its radioactive decay, it is almost absent from fossil products older than 20 000 to 30 000 years. The ^{14}C content may thus be considered a tracer of chemicals recently synthesized from atmospheric CO_2, particularly of recently produced products. It is also the base of the carbon dating method.

Prof. Narayan from the University of Michigan was the first to propose to use the presence of ^{14}C to determine the content of bio-based material in a product. His work led to the development of an ASTM Standard Test Method based on the quantification of ^{14}C carbon called ASTM D-6866. This approach has the advantage of being simple and easy to implement. Also focusing on carbon can be of interest in the context of carbon footprint or carbon dioxide production evaluation. But when trying to communicate on the content of biomass or renewable resources in a given product, this method needs to be completed.

As a matter of fact bio-based materials and compounds also contain large quantities of other elements. These are primarily oxygen, nitrogen, hydrogen and others, coming from lipids, proteins and carbohydrates. These elements are not covered by the radiocarbon method, which in fact leads to the determination of the "bio-based carbon content" in a product. The term "bio-based content" should be used in the meaning of bio-based material including the chemical elements mentioned above.

In 2010 an expert group of the Association de la Chimie du Végétal (ACDV) started working on the development of a method of determination of the bio-based content. Their work was based on the following principals. The approach using isotopic carbon to determine the bio-based carbon content of a sample cannot be applied to other elements such as oxygen, nitrogen or hydrogen, since the half-life value of their radioactive elements is not adapted. A direct determination is therefore not possible. However the content of each element can be determined by elemental analysis which leads to the total content of each element without differentiating between the bio-based or fossil origin.

The expert group decided to propose a method consisting in a statement from the producer on the bio-based content of the product, which is then compared with the combined results of

the radiocarbon and elemental analysis and can be validated. The method was further developed by a CEN working group within CEN/TC 411 on bio-based products as prEN 16785-1, *Bio-based products – Bio-based content – Part 1: Determination of the bio-based content using the radiocarbon analysis and elemental analysis*. It was recently validated by a Round Robin test. Results were published in the beginning of 2016.

However the significant cost of ^{14}C measurement is a disadvantage for regular quality control. Therefore it could be interesting to investigate other potential methodologies. Isotopic ratio analysis could be an alternative method. This report is dedicated to assess the direct automation for isotopic ratio determination: equipment, methods and performances.

3 State of the art on Biobased content and isotopic approaches

3.1 Determination of Biobased content using ^{14}C method: principle, advantage and limitations

Biobased products are products which are wholly or partly made from biomass. They could be feedstock materials, semi-finished components or final commercial products. The determination of biobased content (amount of biomass content) has been undertaken using the determination of ^{14}C isotope. (Another radioactive organic element Tritium (^{3}H) can be used for recent dating especially for groundwater, but has not yet been investigated in the biobased content approach).

There are 3 isotopes of carbon: ^{12}C and ^{13}C are stable and represent the main part of natural occurring carbon whereas ^{14}C is a radioelement (beta particle emitter) and founded in trace amounts. Due to the radioactive decay of ^{14}C, the measurement of the radio activity of ^{14}C allows clearly the differentiation of fossil compound origins to the natural and renewable organic resources. Plants fix CO_2 during the photosynthesis process so the level of ^{14}C of plant and animal are close to the ^{14}C of atmosphere. On the contrary the ^{14}C is absent of fossil originated components. The consequence is the ^{14}C amount could be efficient to determine the part of fossil origin and natural renewable origin in compounds and by the way the Biobased content.

Two main techniques are available for the determination of ^{14}C

-AMS – Accelerator Mass Spectrometry

-LSC – Liquid Scintillation Counting

Unfortunately this approach based on ^{14}C isotopic measurement is only feasible for carbon but not for the other organic elements contained in biomass: hydrogen, oxygen and nitrogen. The result is the determination of Biobased carbon content. In order to improve the approach of biobased carbon content based on ^{14}C determination, the European Standardization worked on a new methodology coming from the French association ACDV based on the determination of ^{14}C and the elemental analyses of the organic elements: ^{14}C + %C + %H + %N + %O. The amount of organic elements is determined using elemental analysers based on mineralisation process (combustion or pyrolysis) transformation of the interested elements into gas and quantified using dedicated detectors. This technique has been developed into EN 16785-2: *Bio-based products — Bio-based content — Part 2: Determination of the bio-based content using the radiocarbon analysis and elemental analysis.*

The determination of biobased content could be undertaken on gas, liquid and solids. Products have been divided in two groups:

-products obtained by chemical synthesis

-products made by formulation (addition of several constituents)

The biobased content of products made by chemical synthesis is undertaken using the approach previously described (^{14}C + CHNO %). For the formulated products that can include several constituents, it is not possible to use this approach with efficiency. In that case the ^{14}C is the only determination performed and the result is used to confirm the Biobased content given by the manufacturer.

The biobased content of natural product is equal at 100% and it is not necessary to verify it. If the reactant is a fossil origin the biobased content is equal to 0%. The biobased content of a component made by a mix of different resources (plant and fossil feedstock) will be represented by the proportion of the different origins. The method is quite efficient and has been validated on different types of compounds with success (1).

The biobased carbon content method and the Biobased content methods are very useful tools developed for the industrial market. They will be the reference method for the assessment of the biobased product of an industrial process. But these methods encounter some limitations. For instance, if the biobased content is linked to the organic element (carbon, hydrogen, oxygen and nitrogen) the information concerning the origin is only given by the ^{14}C radioactive isotope and there are no equivalents from the other organic elements.

The development of the biobased industry will continues in the near future in relation to the current economic and social issues: resources are renewable and sustainable, biomass is a trap of CO_2 and has no effect of increasing CO_2 levels in atmosphere and has thus a beneficial action on climate change. Green industry cannot be relocated and will bring health and employments on the European continent. Regarding these different facts, it has been evident that the checking and verification of biobased content of various products (raw materials, semi-finished components, and commercial products) will increase significantly in the future. But in the course of the development of an industrial biobased market leading to a significant number of transactions of products, some problems could appear:

a. The cost of ^{14}C analysis (few hundreds of euros) is relatively expensive reported to regular monitoring of raw materials.
b. This analytical control cost would be significant for small or middle size companies and they probably would have difficulties to guarantee the biobased origin of the raw materials purchased to be incorporated in their industrial process.
c. Currently there is a limited number of ^{14}C laboratories involved in analysis control in Europe and even in the world.
d. The time required to check the assessment of Biobased content could be important in time (a few days) and the time required to give an answer could be inappropriate in case of regular trades.
e. The fastest method for ^{14}C measurement is the Accelerator Mass Spectrometry method but the price of this equipment is high (close to one million of euros) and the decision to invest in this equipment is not easy to take.
f. The ^{14}C laboratories are not necessarily experts in organic elemental analyses, and they don't always have the dedicated devices. In order to obtain the determination of bi-

obased content several laboratories would have to collaborate thus leading to an increase of the time required.

g. The ^{14}C determination need also to have the $^{13}C/^{12}C$ measurement performed otherwise there could be a bias on the result.
h. The ^{14}C is only limited to the determination of the biobased content of the whole product and cannot distinguish the origin of the molecules in a mixture.

3.2 Interest of stable isotope approach to be an alternative method

Isotopes are defined as atoms of the one element that have the same number of proton and differ in the number of neutrons present in their nucleus.
For example carbon 13 is an isotope of carbon 12. The atomic number of carbon is 6, which means that every carbon atoms has 6 protons and 6 electrons and the neutron number is 6 for carbon 12 and 7 for carbon 13.

Element	Isotope	relative abundance (%)	Mass
Hydrogen	^{1}H	99.985	1.0078250
	^{2}H	0.015	2.0141020
Carbon	^{12}C	98.900	12.000000
	^{13}C	1.100	13.003355
Nitrogen	^{14}N	99.630	14.003074
	^{15}N	0.370	15.000109
Oxygen	^{16}O	99.762	15.994915
	^{17}O	0.038	16.999130
	^{18}O	0.20	17.999159
Sulphur	^{32}S	95.020	31.972072
	^{34}S	4.120	33.967868

Table: Relative abundance data and masses for organic elements and isotopes
Isotopic values are expressed in the Delta unit (δ), according to the following formula:

$$\delta(^{13}C/^{12}C) = \left[\frac{R\,(^{13}C/^{12}C)sample}{R\,(^{13}C/^{12}C)standard} - 1\right] * 1000$$

The delta is expressed in relation to the international standard specified for every isotope
Vienna Pee Dee Belemnite (V.P.D.B.) for $^{13}C/^{12}C$
Vienna Standard Mean Ocean Water (V.S.M.O.W.) for $^{2}H/^{1}H$ and $^{18}O/^{16}O$
Nitrogen of Air for $^{15}N/^{14}N$

However stable isotope analyses are carried out using an internal standard, usually a gas bottle (CO_2, H_2, CO or N_2) regularly injected during the analysis via an Inlet system or an online interface and previously calibrated to the international reference standard.

Biomass is made from C, H and O elements. The $^{13}C/^{12}C$ ratios of plants is mainly affected by the botanical origin of the plant and dependant on the photosynthetic pathway (discrimination among C3 (Calvin cycle), C4 Hatch-Slack cycle) and CAM (Crassulacean Acid Metabolism) photosynthetic cycle origin) and by several factors linked to their position and physiological factors. The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ isotopic composition plant material are linked to the environmental and geographical position (latitude, altitude) and climatic conditions (temperature, precipitation humidity…). Every element disposes one organic isotope that can bring information. Isotopic variations come from their origins and the metabolism of plants but also related manufacturing processes. Isotopes variations can occur during chemical reactions and biological mechanisms.

Isotopic measurements are performed using an Isotope Ratio Mass Spectrometer (IRMS). In order to take into account different samples (solids, liquids), automatic elemental analysers are connected to isotope ratio mass spectrometer for bulk sample materiel usually called Bulk Stable Isotope Analysis (BSIA) or using separate methods for Compound Specific Isotope Analysis (CSIA). In this CSIA approach, isotopic ratios of individual components are determined in a mixture sample.

The bulk stable isotope analysis is easy to operate, carries out analyses in few minutes, has a relative low cost and provides elemental amount of the target elements and multi isotopic determinations. This approach is extremely appropriate for regular authenticity control of natural product and is well used in this scheme. Usually isotopic instruments are able to give the isotopic values of all organic elements contained in the samples.

The separate methods undertaken for Compound specific isotope analysis work in few hours and permit to treat elaborated mixed chemical products. The development of natural product trading required from the industry to be sure of the quality of the raw material they purchase. A significant number of analytical methods were developed following the evolution and the improvement of instrumentation.

3.3 Evolving and prospects of the stable isotopic instrumentation

In 1939 mass spectrometric measurements of natural isotopic variations began with the work of Nier and Gulbransen (2). Urey introduced a dual inlet system in 1948 (3) for the direct introduction of gas into isotope ratio mass spectrometer which was an important breakthrough for new investigations. In 1950 Mc Kinney et al. (4) modified the previous mass spectrometer designed to increase the precision in the measurement of relative abundance by an order of magnitude. Following this work Craig (5) published the required correction factors used to eliminate the inherent error due to the contribution of ^{17}O isotope on carbon dioxide.

During several decades the combustion of targeted molecules was done only by offline devices and the CO_2 recovered introduced manually via the inlet system.

Isotopic users quickly understood the importance of operating using automatic methods due to the elevated number of samples to be taken into account. Researches were then undertaken to develop direct automation using hot mineralisation devices to be connected to the isotope ratio mass spectrometers (EA-IRMS). These instruments appear in the late 1980s.

Steven et al (6) present $\delta^{13}C$ results of research on honeys for authentication made on elemental analyser connected to a mass spectrometer (ANCA-MS). The system includes 3 parts: an automated Dumas combustion, chemical and GC purification of sample-derived CO_2 and isotope analysis by mass spectrometry. Martin et al (7) develop the method for authentication of fruit juices. $\delta^{13}C$ of ethanol and sugars were obtained with a Finnigan Delta E mass spectrometer equipped with a Carlo Erba micro-analyser. In 1992 a newly automatic instrument was developed for analysing $\delta^{13}C$ and $\delta^{15}N$ capable of taking into account 30 samples per day. A C.H.N. rapid elemental analyser Heraus was connected to an isotope ratio mass spectrometer Finnigan Delta E. The CO_2 and N_2 targeted gas were separated using cryogenic traps (8) .

The development of the GC-combustion-IRMS occurred in the beginning of 1990 (9). After separation by capillary GC, the eluting compounds are oxidized in a cupric oxide packed combustion furnace at 850°c in a flow of helium. Water is removed using Nafion membrane and the CO_2 is conveyed in the isotope ratio mass spectrometer. In 1994 (10) Brenna present an automatic coupling made by GC-Combustion interface–IRMS for rapid and convenient compound specific isotope analysis which contained the base of the modern devices. In 1999 Isotope ratio of $^{2}H/^{1}H$ made by high resolution gas chromatography-mass spectrometry (HRGC-IRMS) using a pyrolysis reaction was introduced (11) .Hydrogen contained in the separated molecules is converted into H_2 at temperature over 1400°c in a helium stream. The uncertainty is close to 2δ and the amount of sampling is in the range 200-300 ng.

The same approach was published for the determination of $^{18}O/^{16}O$ ratio on EA-IRMS. Samples were decomposed at 1400°c in a pyrolysis unit containing nickelized carbon half fill in a glassy carbon. (12) Oxygen contained in the sample was converted into CO and measured using the 28 and 30 cups of the isotope ratio mass spectrometer. A similar method was published in 2001 (13) . A continuous flow isotope ratio mass spectrometer is connected with a high temperature pyrolysis elemental analyser. Hydrogen of organically compounds is turned into H_2 in a flow of helium on chromium reactant hold at 1100°C. In 2011 Fourel et al. (14) improved the $\delta^{18}O$ method applied on various organic or inorganic materials by adding a new “purge and trap” module on the Vario-PYRO-Cube (Elementar Hanau Germany). This elemental analyser hold at 1450°c operates using glassy carbon in the pyrolysis unit. Several inorganic matrices (sulphates, nitrates and phosphates were taken into account with success. The combination of an elemental analyser based on combustion and purge and trap and connected to an IRMS was also applied with success for the determination of $\delta^{15}N$, $\delta^{13}C$

and $\delta^{34}S$ simultaneously (15). Loader et al (16) developed also a triple isotope approach ($\delta^{2}H$, $\delta^{13}C$ and $\delta^{18}O$) using an equipment made by an automated unit Interface connected with an elemental analyser and an isotope ratio mass spectrometer. Performances were evaluated through cellulose reference materials compared with classical alternative methods. Results were in good accordance.

Development of GC-combustion-pyrolysis-IRMS has been also undertaken regarding the different possibilities of sampling and injections. Hattori (17) presented a method allowing the isotopic determination of ethanol in alcoholic and non-alcoholic beverages with Head-Space Solid-Phase Micro-Extraction (HS-SPME). HS-SPME is used as a sample preparation technique for the extraction of volatiles from the sample. This approach enables the determination of $\delta^{13}C$ and $\delta^{2}H$ values in aqueous solutions at micromole levels and in higher viscosity matrix samples without an isotopic discrimination. Schipilliti et al (18) investigated a wide range of other selected volatile molecules by HS-SPME connected to GC-C-IRMS. The results were in good agreement with those obtained by extraction enabling this method to be operational for quality control. Shouakar et al (19) applied the GC-pyrolysis (chromium)-IRMS to chlorinated solvents (trichloroethene and dichloroethene) for the detection of $\delta^{2}H$ ratios. The accuracy of the method was evaluated by comparing with offline preparation.

One major challenge for application of CSIA is the analysis of low concentration of organic compounds. Isotope requires sufficient materials to provide an accurate measurement of isotope ratios. Herrero-Martin et al (20) present an implement allowing a pre-concentration technique (headspace auto-sampler connected with a programmed temperature vaporizer) connected to GC-C/P-IRMS for $\delta^{13}C$ and $\delta^{2}H$ isotope analysis. The method enables the isotopic determination of volatile organic constituents at lower concentrations (tens of micrograms per liter) in water.

GC-IRMS offer the possibility for isotopic measurements of one gas into mix. Jia et al. (21) proposed optimal parameters dedicated for the determination of $\delta^{2}H$ into natural gas (methane, ethane and propane) with good precision.

Currently the last commercial evolution of GC-C/P-IRMS incorporates a quadrupole mass spectrometer directly connected onto the equipment.

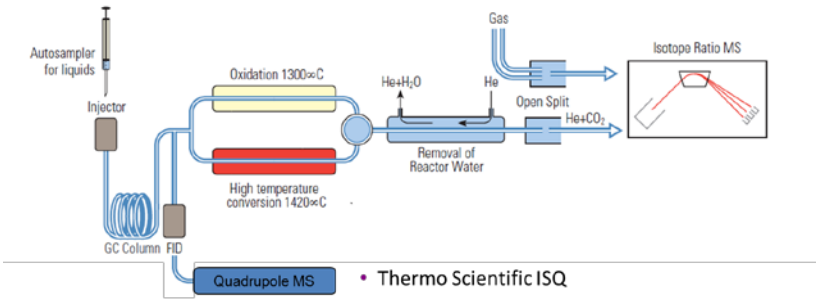


Figure: schematic GC-SM-C/P-IRMS

A T-piece placed after the GC column permits to divide the single injection in two parts: the structure of the molecule (identification of unknown product) and isotope ratio of each compound can be determined.

The gas chromatography –isotope ratio mass spectrometry (GC-C-IRMS) has been the most commonly used technique for compound specific isotope analysis of carbohydrates, drugs, steroids … However a large number of theses samples have to be derivatised to make them volatile and separable in GC-IRMS. Due to the addition of carbon atoms from the derivatization reagents the $\delta^{13}C$ of the targeted molecules are altered and a correction must be done and decrease the accuracy.

In 2004, a new interface has been elaborated for the online determination of $\delta^{13}C$ of molecules using a chemical oxidation module placed between a liquid chromatography and the isotope ratio mass spectrometer: LC-co-IRMS (22). Carbon contained in the organic molecules is converted into carbon dioxide using a mix of oxidant and acid (H_3PO_4 and $Na_2S_2O_8$). CO_2 is extracted from the liquid phase, partly dried on Nafion membranes and introduced via an open split in the source of the isotope ratio of mass spectrometer. This implement will allowed new investigation on components only separated using the different various models of HPLC and permitted to analyse components without the derivatization step. However the organic solvents classically used in chromatography (methanol or acetonitrile) are prohibited in the mobile phase because they will generate a high level of background and cannot permit to obtain efficient results. Most of the research works are focused on the determination of the origin of various carbohydrates to distinguish from C4 plant origin (corn, cane) C3 plants (beet…) and synthesis origins.

Improvements have been done using high temperature liquid chromatography (HT-LC) on separation. The polarity of water can be changed by applying temperature gradients and organic solvents include in the mobile phase are not necessary required in order to obtain the same performances. Zhang et al (23) present a method of the determination of $\delta^{13}C$ using HT-LC/ detector PDA/IRMS for non-polar free steroids. The good separation avoids isotope fractionation and permits to obtain sufficient accuracy.

The development of Position-Specific Isotope Analysis (PSIA) using Pyrolyseur GC-IRMS is a recent methodology allowing the determination of intramolecular carbon isotope measurements. Yamada and al. (24) reported a method able to measure $\delta^{13}C$ methyl and $\delta^{13}C$ carboxyl in addition to the $\delta^{13}C$ acetic acid. Hattori et al (25) improve this approach in vinegar using equipment made by: GC-pyrolysis-GC-C-IRMS.

Oba and Naraoka (26) determined $\delta^{13}C$ site specific carbon isotope analysis for non-volatile aromatic carboxylic acid using partial pyrolysis prior to the isotopic determination. Maning et al. (27) offered new possibilities to distinguish $\delta^{13}C$ from different components using Thermogravimetric Analysis linked to Isotope Ratio Mass Spectrometer (TG-IRMS). This ap-

proach provides new benefits to determine $\delta^{13}C$ of heterogenic samples (mix of C4 and C3 plant origins).

Position Specific carbon isotope analysis of trichloroacetic acid has been performed on GC-C-IRMS (28). The method is based on degradation of trichloroacetic acid into chloroform and CO_2 by thermal decarboxylation and has been demonstrated that it could be potentially used as a routine method. Gilbert et al (29) evaluated the accuracy of headspace solid phase microextraction (HS-SPME) combined with an online pyrolysis system and connected to GC-C-IRMS. The investigation was done on the different fragment (CO, CH_4 and C_2H_4) of ethanol sample with various origins (maize, cane, beet, fossil…). The combination of classical molecular approach and intramolecular methods constitute a powerful tool for the authentication of origin of alcoholic and organic acids products.

The spectroscopic approaches offer an attractive alternative to mass spectrometric detection of isotopologues. Isotope Ratio Infrared spectroscopy (IRIS) is based on an absorption path length of several kilometres achieved with high-reflectivity mirrors that keep the laser beam inside cavity for a large numbers of reflections. This emerging technique currently enables the isotopic ratio analysis of (^2H /^1H, ^{13}C ^{12}C, ^{15}N/^{14}N and ^{18}O/^{16}O) on different gaseous molecules (CO_2, CH_4, H_2O, N_2O …). Two major suppliers develop spectroscopic analysers: WS-CRDS (Wavelength-Scanned Cavity Ring-Down Spectroscopy) by Picarro (Inc., Sunnyvale, California) and OA-ICOS (Off-Axis Integrated Cavity Output Spectroscopy) developed by Los Gatos Research (Mountain View, California).

Compound specific isotope analysis of isotope ratio by elemental analyser connected to laser detector is in the early development stage. Koehler et al (30) present a coupling made by an elemental analyser connected to a laser spectrometer (EA/OA-ICOS) for the determination of δ^2H ratio. The combustion is done at 1000°c in flow of air in the elemental analyser and the determination of δ^2H is done of the H_2O following the combustion of H contained in organic molecules. Picarro Inc. proposes a commercial device suitable for the determination of $\delta^{13}C$ ratios made by a combustion module connected to a Cavity Ring Down Spectroscopy detector (CM-CRDS). The combustion is done in pure oxygen and nitrogen flow is added prior to be introduced in the laser.

In summary various ranges of automatic analytical techniques has been developed and improved for the determination of the isotopic ratios since a few decades. Bulk stable isotope analyses made by EA-IRMS and Compound specific isotope analyses using GC-C/P-IRMS or LC-co-IRMS are certainly the main tools studied. All of the investigations permit to gain in accuracy, in performances increasing the possibility of application and the field investigated. The multi isotopic approach could be carried out in the same run or made by two fast analysers. These performances permit an increasing of isotopic results investigated in a short period of time and give the possibility to have supplemented isotopic data rapidly that can help to improve the diagnosis of the origin of Biobased compounds assessment.

Furthermore the last innovations in Position Specific Isotope Analysis approach offer the possibility to obtain supplementary intramolecular data related to targeted molecules. Current researches are focused on the different devices capable to break the molecules in various fragments prior to be injected for compound specific isotope analysis.

In parallel the development of new devices based on isotope ratio infrared spectroscopy (IRIS) is a new field of research. These isotopic analysers operate under O_2 or air flow and the development of coupling method (EA-IRIS) for BSIA approach will probably permit new isotopic approaches in the upcoming future.

4 Experimentation: Instrumentation and Methods

Isotopic measurements are performed on gases using an Isotope Ratio Mass Spectrometer. Two isotope ratio mass spectrometers linked to different external modules have been involved in the European project Open Bio.

A THERMOFISHER Delta V Plus mass spectrometer

An ELEMENTAR Isoprime mass spectrometer

These isotope ratio mass spectrometers are modern devices and representative of the equipment found in the isotopic laboratories.

Isotopic determinations could be performed in three options:

- Inlet system for gases directly measured.
- Continuous flow for bulk sample isotope analysis
- Continuous flow for compound specific isotope analysis

4.1 Isotope Ratio Mass Spectrometer: Delta V Plus and Isoprime

Isotope Ratio Mass Spectrometer (I.R.M.S) is a mass spectrometer dedicated for the determination of stable organic isotope ratios. This device is able to produce precise, stable and accurate measurements on gas sample of the natural abundance of isotopes.

4.1.1 Delta V Mass Spectrometer

The Delta V Plus device is a mass spectrometer characterized by highest sensitivity and linearity and stability. It can receive 10 collectors in the mass range m/z = 96. It is primarily designed to measure the isotope $^{2}H/^{1}H$, $^{13}C/^{12}C$, ^{15}N /^{14}N and $^{18}O/^{16}O$. Delta V Plus can be equipped with a dual inlet system for the determination of pure gases and can be connected with different devices for B.S.I.A. or C.S.I.A.

Isotope ratio mass spectrometer operates under high vacuum. The instrument is usually equipped with a pumping system including one of few primary pumps and one or few turbomolecular pumps.

Gaseous sample to be analysed is introduced into the ion source via an interface or an inlet system. Ions are generated by an impact of electrons in a high vacuum (close to 10-6 mbar). The ionizing electrons are emitted by a thermionic cathode. Ions are accelerated by a 3Kv high voltage and focalized through different lenses hold at different high voltages to form a beam. Two permanent magnets placed at the opposite position to the ionization chamber generating a magnetic field parallel to the electron beam. Due to the magnetic field, the electrons are focused. The energy of the ionizing electrons is determined by the potential difference between cathode and ionization chamber. It has a range between 70 eV and 124 eV. Electron beam is collected in electron trap (anode) located at the opposite of the cathode.

Extraction plates accelerate ions out of the ionization chamber. The following lens system of different lens focuses the ion beam onto the ion source slit.

The ion beam exits the source into the magnetic field generated by an electromagnet with a maximum field of 0,75 tesla. The mass setting is achieved by the variation of the two parameters magnetic field strength and accelerating voltage in accordance with the following equation:

$$M/z = (r^2/2V) * B$$

where:

M is the mass of ion
z is the number of charge of ion
r is the radius of ion path
V is the accelerating voltage
B is the magnetic field value

Several configurations of detectors (collectors) are available for the detection of N_2, O_2, CO, CO_2 ions (universal triple collector) and located within the collector system housing. For HD isotope analysis, a collector system including 2 faradays cup and amplifiers is installed into the same housing. The HD collector system is dual faraday collector assembly for hydrogen isotope measurements on the same ion path.

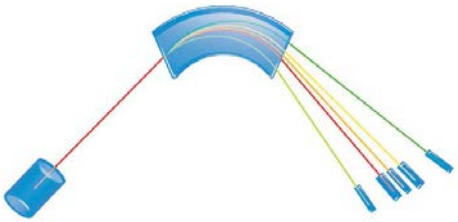


Figure: Universal CHNOS detector (Thermofisher)

Each collector cup has its own amplifier and the feedback resistor of the amplifier can be matched to the abundance of the isotope to be collected in the cup. The collector systems cover the mass range from 10 to 96 AMU (atomic mass unit) at 3 kV accelerating voltage, allowing a resolution of m/Δm= 110 (10% valley).

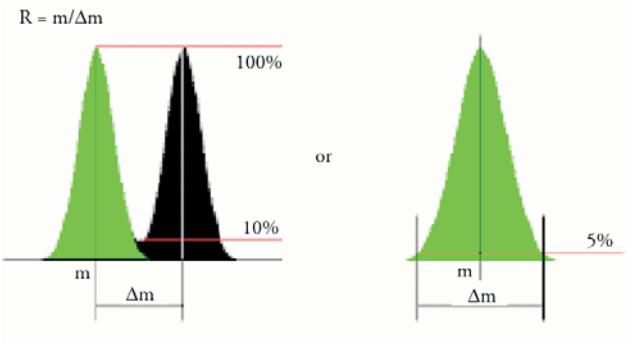


Figure: Resolution of the mass spectrometer (Thermofisher)

Table: Collector arrangement and masses (Thermofisher)

Gas	Collector arrangements for masses (m/z)									
H_2	2									3
N_2			28	29	30					
CO			28	29	30					
NO			30	31	32					
O_2			32	33	34					
CO_2			44	45	46					
N_2O			44	45	46					
SO_2			64	66						
Delta V Plus- additional Collector Arrangements										
Air	28	29	32	33	34	36	40	44	45	46
N^2O	28	29	30	31	32			44	45	46
CO,CO_2	28	29	30					44	45	46
N_2,CO_2	28	29	30					44	45	46
SO_2								64	65	66
SO,SO_2					48	49	50	64	65	66
CH_3Cl					50	51	52	53		
CH_3Br									94	96

The output voltage is the product of the input current and a feedback resistor. The feedback resistor must match the abundance of the isotope to be collected in the respective collector cup. Isotope ratio mass spectrometers are able to take into account a wide range of organic gases for isotope ratio measurements including organic-halogen gases.

The table below shows the resistor values installed for isotopes of the different gases

Table: Resistor values matching natural abundances of isotopes

Gas	m/z	amplification
H2	2	1.10^{9}
	3	1.10^{12}
N2	28	1.10^{8}
	29	1.10^{10}
	30	1.10^{11}
CO2	44	1.10^{8}
	45	1.10^{10}
	46	1.10^{11}

4.1.2 Isoprime mass spectrometer

Isoprime is an Isotope Ratio Mass Spectrometer operating within the same principle as the IRMS Delta V Plus previously described. The major components are an ion source (type electron impact) an analyser equipped with a magnet covering a mass range of 20-70 AMU and a triple collector system for the detection. Isoprime include also the hydrogen collector with electrostatic filter allowing measurements of $^{2}H/^{1}H$ isotopes. Molecules are ionized into the source, focused and formed into a bean, accelerated by an electric field, deflected in a magnetic field and finally detected using collectors. Isoprime operate under high vacuum using a pumping system made by a rotary pump (primary pump) a turbomolecular pump allowing to reach high vacuum (10-9 mbar).

4.1.3 Inlet system

Both gases (reference/ sample) are introduced in variable bellows via a system of valves. Before entering the ion source on an IRMS, the analyte must be converted into a simple gas, isotopically representative of the original sample .Samples to be analysed using Inlet system are previously prepared by offline process and the selected molecules are converted into gases. Usually the offline process is done with pumping system. The Inlet system allows ultimate precision and accuracy by close comparison of sample and reference gases under the same physical conditions.

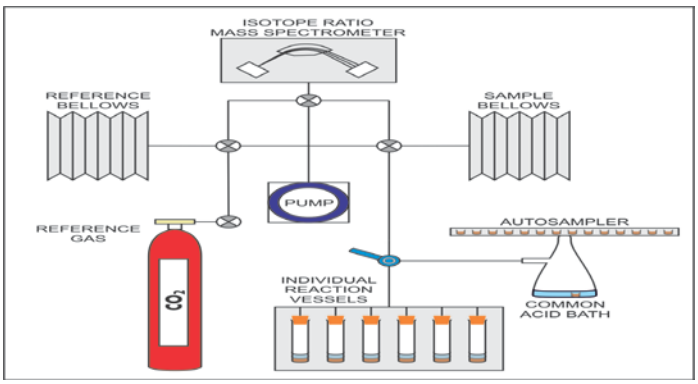


Figure: Inlet System – operating system

The Inlet system is a specific module made with all-metal valve blocks and use gold-sealed and gold-seated valves. The changeover valve is mounted directly to the ion source housing, a design with minimal dead volume and minimum gas path length. The variable volumes (bellows) can be adjusted between 3 and 40 millilitres. Each bellows is monitored to set same sample-standard pressure balancing to ensure the highest level of performance. The excess of gas of the waste line is pumped by a turbo-molecular pump. The Inlet system, including the capillaries, has integrated heating in order to eliminate water and minimize memory.

4.2 Continuous flow IRMS (CF-IRMS)

4.2.1 Online interface

The online interface is an interface dedicated for all online continuous flow peripherals to the gas isotope ratio mass spectrometer. It permits to connect the different peripherals (Elemental analyser, GC-Combustion/pyrolysis. The online interface has 2 missions:

Open split
Sample/standard referencing

Open split

Prior to the isotopic measurements performed in IRMS the samples must converted into simple gases. Usually this is accomplished by oxidation, reduction or high temperature conversion. The resulting gases H_2, N_2, CO, CO_2 and SO_2 can be introduced in the source of the IRMS. The online interface is equipped with on open split interface (small capillary) to keep the same pressure in the source and introduce a small but stable part of the helium flow in the IRMS (0.3 mL/min from 80 mL/min).

Sample /standard referencing

The high precision isotope ratio needs to be referenced to a calibrated standards gas in order to minimize effects of instrumental drifts, it is recommended to measure the calibrated gas just before or after the sample gas peak. The online interface provides the connection of every reference gases required (H, CO_2, CO, N_2 and SO_2). The reference gas is introduced in the IRMS via a separate capillary. A diluting system with helium permits to select the level of the gas standard.

The linearity test requires to measure a serie of reference gas pulses with different intensities.

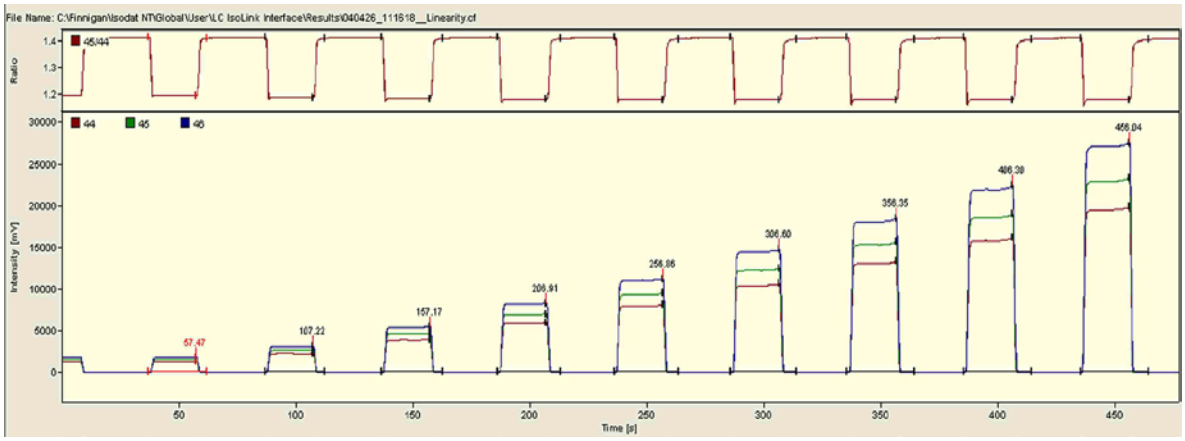


Figure: Chromatogram of the linearity test

Table: Linearity test- table of results and variability of $\delta^{13}C$ obtained
(The yellow line correspond to the reference standard)

Peak n°	Start (s)	Rt (s)	Ampl 44	**$\delta^{13}C$ measured (‰)**
1	37.2	57.5	1273	**0.244**
2	86.9	107.2	2231	**0.082**
3	136.7	157.2	3927	**0.000**
4	186.4	206.9	5954	**-0.029**
5	236.2	256.9	8062	**-0.040**
6	285.9	306.6	10603	**-0.002**
7	335.7	356.3	13276	**0.047**
8	385.6	405.3	16084	**0.082**
9	435.3	456.0	19837	**0.168**

4.2.2 Bulk Stable Isotope Analysis (B.S.I.A.)

Continuous flow enables to work without preparation, small sizes of sample (close to 1 mg) and fast analyses. Two classical approaches are available:

- Determination of $\delta^{15}N$ and $\delta^{13}C$ performed in combustion mode
- Determination of $\delta^{2}H$ and $\delta^{18}O$ performed in pyrolysis mode

The two devices involved for the isotopic investigation were more or less similar at the other instruments found in isotopic laboratories. The combustion or pyrolysis units are quite similar in operating conditions, any differences could be found for the separation of gas prior to injection in IRMS. Thermofisher use chromatographic devices where Elementar work on trap and purge systems.

4.2.2.1 Thermo Flash EA1112 elemental analyser for δ13C & δ15N isotopic analysis

The Flash EA 1112 is a precise solution to nitrogen and carbon determination based on the flash combustion operation (about 1800°c) suitable organic or inorganic products, solid or liquid samples. Samples sealed in tin capsules drop in the unit combustion hold at 950°c in flow of helium and a short pulse of oxygen. The flash combustion of the sample allows the complete conversion of carbon and nitrogen into CO_2 and NO_x avoiding any matrix effects.

The different gases convey to the reduction furnace where nitrogen is reduced into N_2 and the excess of oxygen is retained, and through a water trap made by anhydrous magnesium perchlorate. CO_2 and N_2 are separated on a gas chromatographic column (GC) and the determination of nitrogen and carbon amounts are performed on a thermal conductivity detector (TCD) in a relative short period of time (close to 5 min).

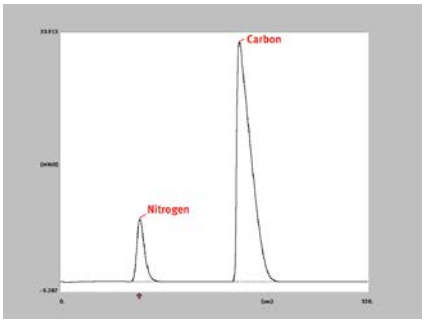


Figure: representation of N_2/ CO_2 separation

The Flash EA 1112 device is usually equipped with a 32 samples tray and 3 additional trays could be added allowing the elemental analyses of several set of samples. An automatic liquid auto-sampler suitable for all configurations could replace the classical auto sampler allowing up to 108 programmed analyses.

The elemental analyser is connected to the isotope ratio mass spectrometer via an online interface (Conflo IV). This intermediary device permits to adjust automatic sample dilution and generation of reference gas pulses, enabling individual referencing of each sample gas peak. A small part of the effluent is introduced into the IRMS via an open split interface (capillary tube).

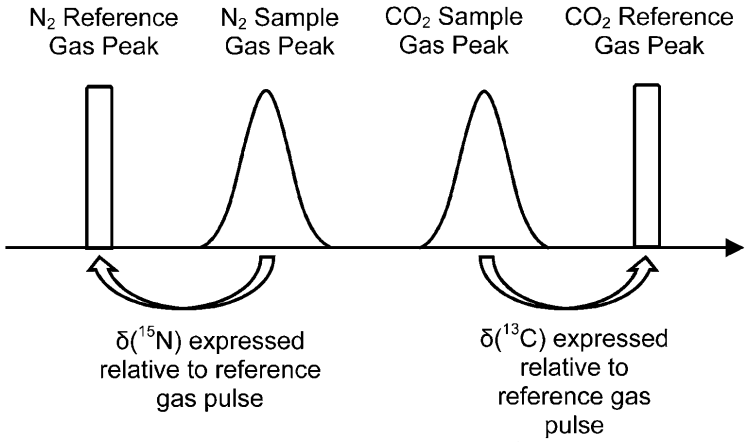


Figure: schematic run of an automatic ^{13}C &^{15}N analyse

The determination of $\delta^{15}N$ and $\delta^{13}C$ is done in few minutes (close to 10 min) allowing measurements of series of samples.

4.2.2.2 Finnigan TC/ EA elementar analyser for δ2H & δ18O isotopic measurements

High temperature conversion elemental analyser (TC/EA) is used for the determination of δ^2H and $\delta^{18}O$ by pyrolysis approach. Samples sealed in silver cups fall into the high temperature pyrolysis unit hold at 1450°c in a flow of helium. The reactor consists of a glassy carbon

tube with glassy carbon filling incorporate on an alumina tube ensuring that no reaction could be done between the sample and the oxygen contain of the inorganic tube (Al_2O_3). This technology permits no memory effect of previous pyrolysis. H and O are converted into H_2 and CO gases, separated on an isothermal gas chromatographic column prior to be introduced in the IRMS via the same interface (Conflo IV).
The interface operates similarly as previously described providing steady and regular reference gas pulses.

Thanks to the high temperature of the pyrolysis unit (1450°) the elemental analyser can be used for simultaneous hydrogen and oxygen isotope ratio measurements of all organic components and any inorganic compounds (sulphates, nitrates, phosphates…).

In order to take into account liquid samples an auto sampler dedicated to liquid injection could be adapted. A special insert will be place in the reactor enabling steady and reproducible injections.

The determination of δ^2H and $\delta^{18}O$ is done in few minutes (close to 6mn) allowing measurements of series of samples.

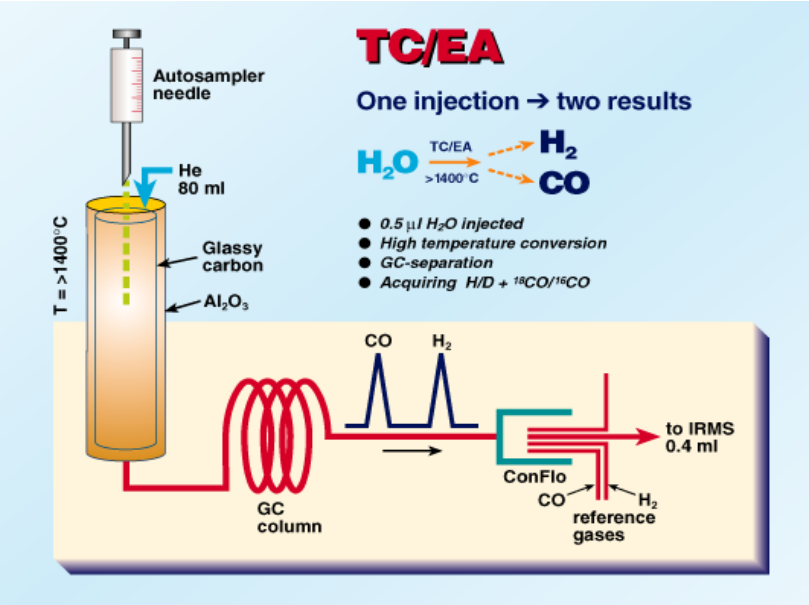


Figure: schematic overview of TC/EA -IRMS

4.2.3 Compound Specific Isotope analysis (C.S.I.A.).

In the compound specific isotope analysis separate instruments are added prior to perform the isotopic analysis. Two types of implements are available:

- -Gas chromatography- combustion/pyrolysis –Isotope ratio mass spectrometer (GC-C/P-IRMS)
- Liquid chromatography-chemical oxidation- Isotope ratio mass spectrometer (LC-co-IRMS)

4.2.3.1 GC-C/P-IRMS

The equipment involved for the investigations was a GC gas chromatograph (6890N Agilent) connected to an isotope ratio mass spectrometer Isoprime (Elementar) via a combustion/ reduction interface (GC5 Elementar)

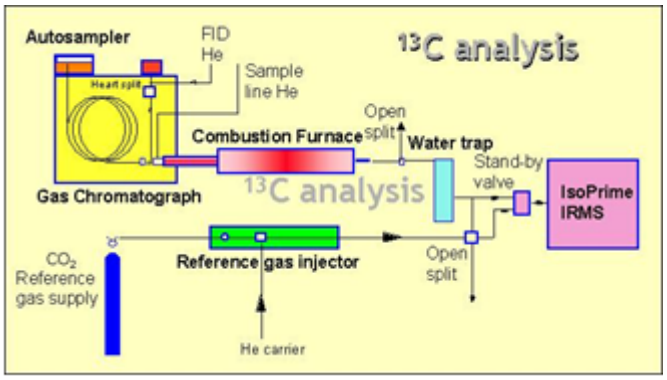


Figure: Schematic GC-combustion–IRMS ISOPRIME

Operating conditions

Samples are injected in the hot injector of a gas chromatograph GC6890N Agilent Technologies in a flow of helium used as carrier gas. Solvent and the different components are separated on a capillary column and the composition of the mixture is determined on the FID detector in a first run.

The second run permits to remote the solvent at relative low temperature to the FID and afterward the targeted molecules are conveyed through the GC5 interface to be transformed according the select isotopic mode:

Determination of $\delta^{13}C$

The interface dedicated to $\delta^{13}C$ measurements is made by a combustion tube half-filled with copper oxide and maintained at 850°c. Carbon contained in the separated molecules is converted into CO_2 gas. Gases are transferred into a Nafion tube used to remove water according the osmose reaction.

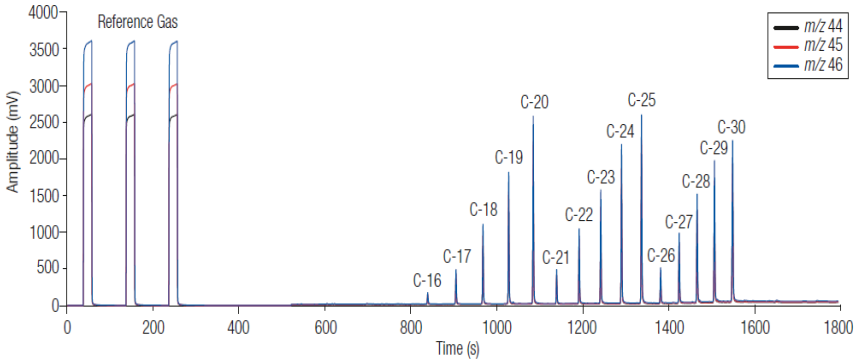


Figure: schematic run paraffin performed on GC-IRMS

Determination of $\delta^{2}H$

The interface consists of a reactor to pyrolyse the compounds, hydrogen contained into the organic molecules are turned into H2. The reactor is a glass tube packed with 15 cm of chromium pellets located into a furnace maintained at 1050°c. Organic samples are pyrolysed according the following reaction:

$$C_xH_yO_z \rightarrow {}_xC + {}_{y/2}H_2 + {}_{Z/2}O_2$$
$$2Cr + {}_{3/2}\ O_2 \rightarrow Cr_2O_3$$

A further contribution due to the mass 3 signal originated from the formation of ion-molecule reactions between H_2^+ and H_2 molecule must be taken into account in the source. Because the contribution depends of the amount of H_2, a correction has been established (H_3^+ factor) calculated within the measurement of different intensities of reference gas. The usual requirement of the H_3^+ factor is a value less than 10 ppm.

For the both equipment the amount of standards and samples amount is close to few milligrams in a mix on one milliliter of solvent.
A classical run is done in one or a few hours and the auto-sampler permits to take into account several samples.

The GC-C/P-IRMS equipment previously described is similar to the other commercial implements. The differences are mainly linked to the pyrolysis unit (t°, reagents) but the performances are similar.

4.2.3.2 LC-co-IRMS

The implement involved for the investigations was an ionic chromatography ICS 5000 (Dionex) connected to an isotope ratio mass spectrometer Delta V plus via an interface of oxidation Isolink (Thermo). A similar commercial equipment supplied by Elementar allows quite performances and operate in the same approach.

The liquid chromatography (LC) Isolink interface is a dedicated device created only for $^{13}C/^{12}C$ isotope ratio applications using HPLC separations. In isotopic configuration the eluent phase couldn't contain organic solvents (methanol, acetonitrile…).

In order to obtain good separations of molecules in liquid modules HPLC was replace by an IC device in our laboratory. Separation of the selected molecules is done using an ionic eluent and these components are eliminated before injected into the chemical interface by an ionic suppressor. Its sensitivity, precision and accuracy enable the compound specific isotope analysis (CSIA) of bioactive components with high polarity and high molecular weight. It offers full automation and maintenance-free operation.

Individual organic compounds in aqueous phase are oxidised into CO_2 using a mix of oxidant peroxodisulfate agents and phosphoric acid. Afterward the mobile phase is separated from the formed CO_2 by the separation unit.

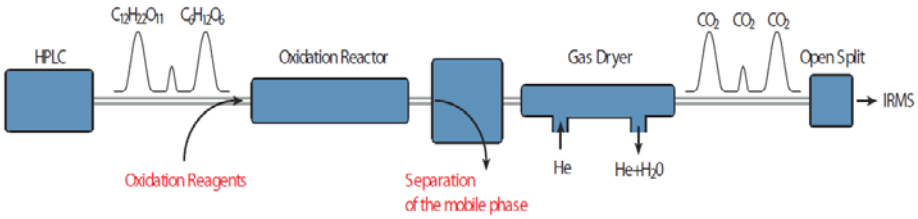


Figure: Schematic HPLC-co-IRMS

The liquid interface can operate in either of two injections mode:

A. Compound specific isotope analysis in HPLC mode.

Samples are injected by a loop injection valve in front of the HPLC column. The mixture of organic compounds in the sample is separated on the HPLC column and the constant flow of the mobile phase is maintained through the oxidation interface.

B. Bulk stable isotope analysis in the Direct Injection mode (µ-EA mode).

The analytical mode is a fast analysis of all water soluble materials and is thus a bulk measurement. Reference materials and unknown bulk samples can be analysed with unmatched sensitivity and speed. Samples can be injected via the needle port into a sample loop of variable size, which is placed at the six-port-valve behind the HPLC column. The mixture of reagents and mobile phase flows through a capillary oxidation reactor hold at 99 ºC. Here all organic compounds are converted individually and quantitatively into CO_2 without isotope fractionation: After passing the oxidation reactor, the mobile phase is cooled. The individual CO_2 peaks are separated from the liquid phase by a separation unit: the CO_2 is transferred through thin membranes into a counter flow of helium. The use of membranes, the pressure difference and the difference in CO2 concentration between inside and outside of the membranes contribute to an almost complete degassing of the liquid phase.

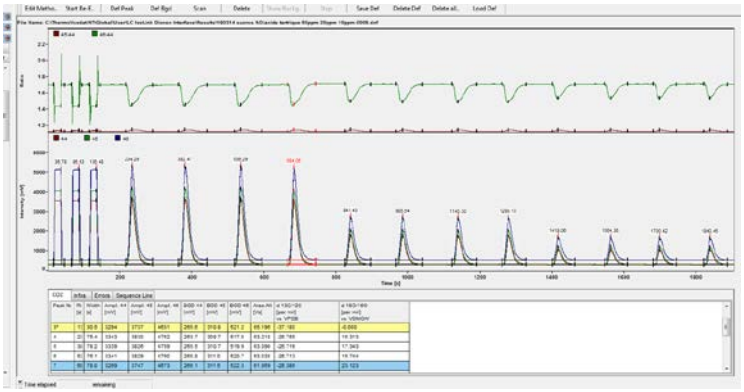


Figure: chromatogram of analyses of tartaric acid in LC-co-IRMS at three levels in BSIA mode

4.3 Performance

4.3.1 Accuracy of stable isotope in pure products

The uncertainty of measurements carried out on modern devices in continuous flow isotope analysis is good and enough to make difference on the difference origins of targeted compounds. The uncertainties given by the supplier for BSIA are close to these values (in delta notation):
$\delta^{13}C$: +/- 0.3 ‰ / $\delta^{15}N$: +/-0.3 ‰ / $\delta^{2}H$: +/- 5 ‰ / $\delta^{18}O$: +/- 1‰

Performance and precision of the different equipment must be verified using references standards. Many international reference standards are supplied by different organisms (International Agency of Atomic Energy, National Bureau of Standards) and validated by intercomparison of several laboratories. Various international referenced standards have been acquired for the determination for bulk stable isotope analysis (EA–IRMS).

Table of the international isotopic reference standard available in the laboratory

References	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{2}H$ (‰)	$\delta^{18}O$ (‰)
IAEA CH-7 (polyethylene foil)	-32.15		-100.3	
IAEA NBS 22 (oil)	-29.79		-120	
IAEA CH6 (sucrose)	-10.45			
IAEA 600 (caffeine)	-27.77	1.0		
IAEA 601 (Benzoic acid)				23.3
IAEA 602 (Benzoic acid)				71.4
USGS 40 (L glutamic acid)	-26.39	-4.5		

However the instruments required to be regularly controlled during the cycle of set of analyses. In the aim two references standard are measured every height samples. Therefore others standards have been purchased and calibrated to the international reference to become secondary reference standards. They are daily measured for ensuring a non-drift of the isotopic operating systems.

Table of the reference standards used in laboratory

Reference	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{2}H$ (‰)	$\delta^{18}O$ (‰)
Valine	-25.88	21.76		
Vanillin	-29.10			
Glucose	-10.79			
Acetanilide	-29.64	-1.49		
Dodecane			-129	
Octanol			-75	
Saccharose	-26.32			
Methyl salicylate				19
Linalyl acetate				11

Statistics have been carried out on isotopic standards with the goal to verify if one or several standards have been altered. The table below collects the isotopic values of reference standards analysed within the last 2 years.

Table of isotopic values determined in EA-IRMS within the last 2 years

references	$\bar{x}$ $\delta^{13}C$ (‰)	SD (‰)	N
IAEA CH7	-32.07 (-32.15)	0.17	229
IAEA CH6	-10.43 (-10.45)	0.21	229
NBS 22	-29.60 (-29.79)	0.19	87
Glucose	-10.76	0.20	161
Saccharose	-26.25	0.20	146
Acetanilide	-29.68	0.20	178
Vanillin	-29.18	0.18	68
$\bar{x}$ Average SD standard deviation N number of measurements			

Standards for C.S.I.A.: (GC-C/P-IRMS)

International standards dedicated to the GC-C/P-IRMS are not available. Due to this fact isotopic values of certain organic reference standards are calibrated to the international standards. Paraffin molecules from C11 to C15 have been selected because they are components only made by C and H and could easily validated the combustion system. The ^{13}C of individual molecule is determined prior to prepare a mixture of these molecules.

The mixture of paraffin molecules is regularly injected in GC-C-IRMS to be sure of the absence of isotopic drift into the operating system.

Table of isotopic results of paraffin molecule determined on EA-IRMS and GC-IRMS

paraffin	$\bar{x}$ $\delta^{13}C$ (EA-IRMS) (‰)	$\bar{x}$ $\delta^{13}C$ (GC-IRMS) (‰)	SD (GC-IRMS)
C11	-27.60	-27.12	0.69
C12	-32.85	-33.02	0.09
C13	-30.01	-29.76	0.10
C14	-30.4	-30.71	0.12
C15	-30.61	-30.89	0.09

Since elemental analysers have been connected to isotope ratio mass spectrometer, they are able to determine the amount of organic element during the running cycle. The amount of CHNO could be linked to the biomass content and thus the uncertainty of the measurements must be evaluated.

Table of uncertainty for elemental organic quantifications

Element	Range	Uncertainty
Carbone	1 - 30 %	0,30 %
	30 - 75 %	0,40 %
	75 - 100 %	0,50 %
Hydrogen	0,30 - 3%	0,20 %
	3 - 15%	0,30 %
Nitrogen	0,30 - 3 %	0,20 %
	3 - 25 %	0,30 %
	25 - 40 %	0,40 %
	> 40 %	0,50 %
Oxygen	0,30 - 20 %	0,30 %
	20 - 40 %	0,40 %
	> 40%	0,50 %

4.3.2 Accuracy of stable isotopes in mixtures

The determination of biobased content can be applied to wholly or partly Biobased products. Thus it would be important to evaluate the precision of the method regarding different levels of mixtures of components.

4.3.2.1 Tests on liquid samples

In order to evaluate the accuracy of stable isotope analysis two model solutions were prepared by mixing compounds in various levels. Isotopic measurements are performed using both EA-IRMS instruments.

Injection 0.1 µL
5 replicates for every level

First test

Establishment of $\delta^{13}C$ calibration curves for butanol (Biobased origin) in solution of gasoline (fossil origin).

Butanol (corn: C4 plant): $\delta^{13}C$: -13.45 ‰
E5 gasoline (fossil origin): $\delta^{13}C$: -27.63 ‰

Samples are prepared by addition of different volumes of the 2 origins

E5 gasoline	butanol
1000 µl	0 µL
950 µL	50 µl
900 µL	100 µl
800 µL	200 µl
500 µL	500 µl
0 µL	1000 µl

Results obtained for the different levels are presented in the following table:

$\delta^{13}C$ (‰)
-27,63
-27,03
-26,43
-25,20
-20,55
-13,45

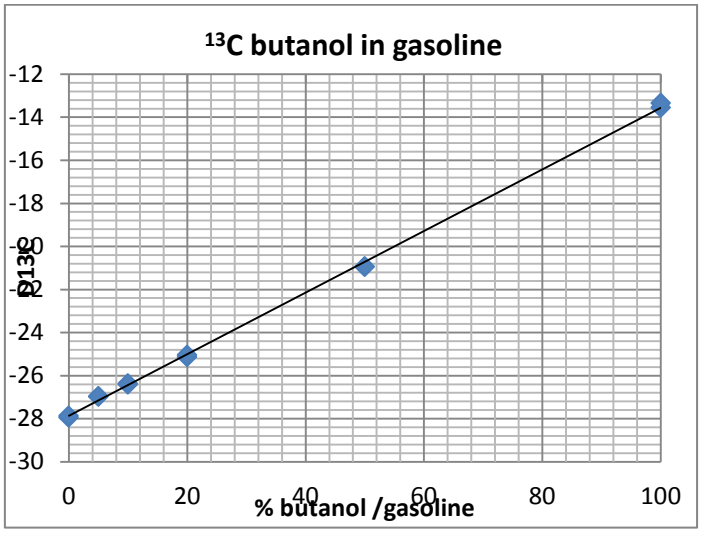


Figure: Regression linear of butanol in gasoline

The extreme points correspond to the pure products. The results obtained present a linear regression among the different levels of the solutions prepared and the isotopic values. The method is efficient for determination with an uncertainty lower than 5%.

<u>Second test</u>

Establishment of δ^2H calibration curves for ethanol (Biobased product) in hydrocracking gasoline (Fossil origin).

Ethanol (sugarcane: C4 plant) δ^2H: -202‰

Gasoline hydrocracking (fossil origin) δ^2H: -83‰

Samples are prepared by addition of different volumes of the 2 origins:

gasoline (volume)	ethanol (volume)
1000 µl	0 µL
950 µL	50 µl
900 µL	100 µl
800 µL	200 µl
250 µL	750 µl
0 µL	1000 µl

Results obtained for the different levels are presented in the following table:

δ^2H (‰)
-83
-89
-92
-96
-176
-202

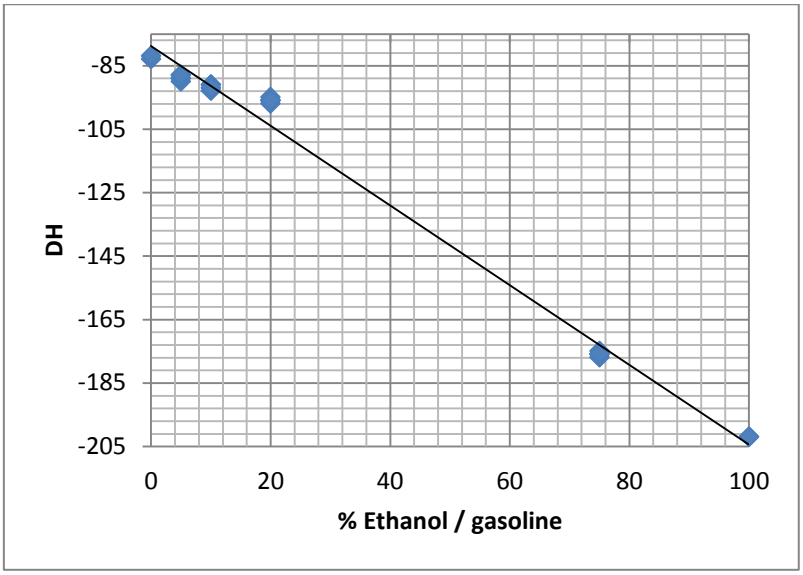


Figure: Regression linear of ethanol in hydrocracking gasoline

The extreme points correspond to the pure products.
The results obtained present a linear regression among the different levels of the solution prepared and the isotopic values. The method is efficient for determination with an uncertainty lower than 10%.

Third example: Mixture of solvents

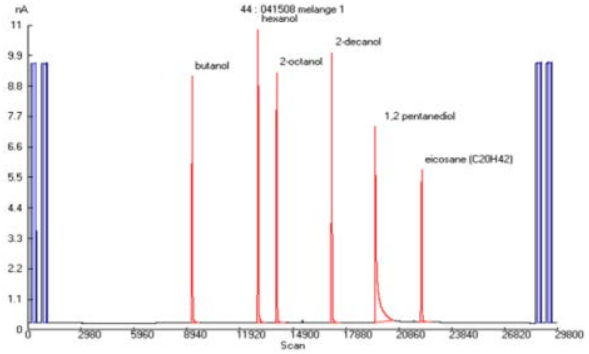
A mixture of biobased and non biobased components has been prepared in order to determine the biobased content of the assembling product.

Table of solvents, $\delta^{13}C$ of pure products and origins

Molecule	origin	$\delta^{13}C$ (‰)	SD
butanol	fossil	-26,80	0,24
hexanol	fossil	-26,13	0,09
2-octanol	fossil	-28,76	0,13
2-decanol	fossil	-34,91	0,11
1,2 Pentanediol	**Biobased**	**-10,24**	**0,05**
eicosan	fossil	-32,78	0,07

The molecule of 1,2 Pentanediol produced on C4 plant (Corn or sugar cane) has a significant fingerprint. However due to a relative high number of products in the mixture, the biobased determination will be made using GC-C-IRMS.

The following chromatogram shows the intensity of the different constituents in the mixture.



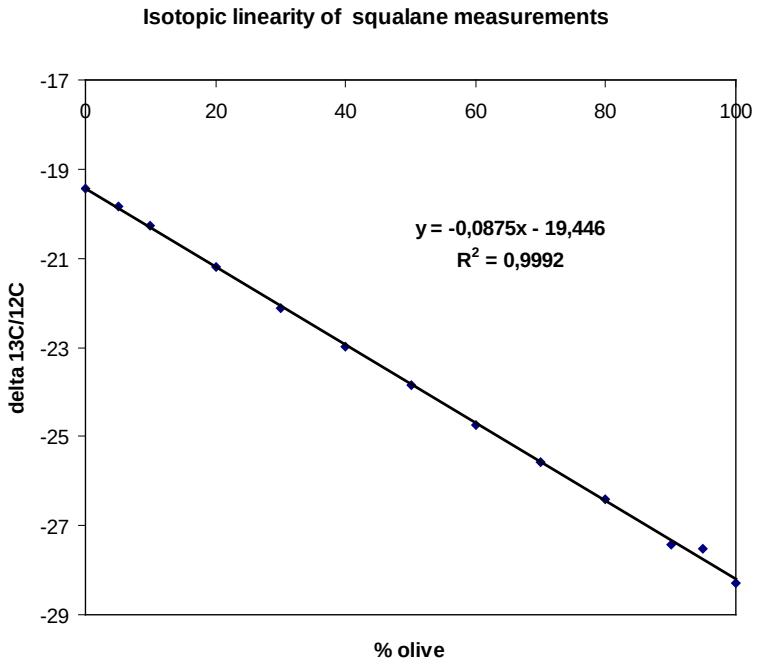
Chromatogram of GC-IRMS of the different constituents included in the mixture

Isotopic values permit to determine which solvent is biobased and non-biobased.
The additional molecular detector (FID or mass spectrometer) include in the GC-C-IRMS permits to known the amount of every constituent in the mixture. Biobased determination of the mixture and on every samples of the mixture could be done with efficiency in that case.

4.3.2.2 Test on non-liquid samples

Assessment of the origin of a cosmetic product as a raw material: squalane.
Squalane is a fully saturated hydrocarbon, $C_{30}H_{62}$. It does not occur in nature. It is a perfectly stable oil and remains fluid at low temperature. Squalane has been widely used in cosmetics for many years. It is an excellent emollient oil and is appreciated for the exceptional silky touch that it brings to cosmetic formulations. Squalane is obtained on an industrial scale by exhaustive catalytic hydrogenation of the naturally occurring molecule – Squalene. Squalene occurs in nature in 2 main areas: shark liver oil (animal origin) and olive oil (plant origin).Both origin are Biobased but the cosmetic industry bans the animal origin from their products and shark couldn't be assimilated as a renewable feedstock.

$\delta^{13}C$ values of squalane olive oil range from -27.8 to -28.4‰ (C3 plant) whereas squalane of shark origin range from -20.9 to -19.9‰. Samples were prepared by adding shark Squalane to olive Squalane in known proportions ranging from 100% olive Squalene to 0% (that is 100% shark Squalane).



An uncertainty of 0.30‰ in the determination of $\delta^{13}C$ values allows the content of olive Squalane to be established within a limit of +/- 4%.

Assessment of the origin of a cosmetic product incorporated in commercial compounds

This a cosmetic cream. As previously described $\delta^{13}C$ is a useful tool for the determination of the origin (plant or animal) of the biobased material squalane. But in the case where squalane has been incorporated in a cosmetic cream, the direct measurement couldn't be done.

The operating conditions required two steps:

- Liquid extraction with organic solvent
- Analysis in Compound Specific Isotope Analysis mode using GC-C-IRMS

The analytical model was validated using a blank commercial cream (without squalane) added with different quantities of the 2 origins. After extractions and analyses, results are presented in the following table.

% olive squalane	% shark squalane	$\delta^{13}C$ measured (‰)	$\delta^{13}C$ calculated (‰)
100	0	-27,9	-27,8
75	25	-25,8	-25,9
62,5	37,5	-25,0	-25,0
50	50	-23,4	-23.8
37,5	62,5	-23,0	-23,1
25	75	-22,1	-22,2
0	100	-20,1	-20,3

Analytical results indicate that no isotopic discrimination has been observed during extraction and analysis. The operating model is efficient to determine the origin of the squalane in commercial cream.

Material made by addition of 2 solid raw materials: composite flax fibre-polyethylene

Composite made by flax fibre and polyethylene are developed for building applications due to a small price, low density and durability propriety. This composite is elaborated as composite pellets and cannot be directly analysed.

In order to obtain a homogenised powder, few pellets are grinded using a cryogenic mill.
Isotopic analyses are firstly performed on the 2 pure products:

Flax fibre $\delta^{13}C$: -28.38‰ 100% Biobased
Polyethylene $\delta^{13}C$: -26.46‰ 0% Biobased

The aim of the test is to determine if isotopic measurements performed on the crushed sample powder are able to bring the biobased content amount. However the gap between both origins is not very high. Due to this fact, several measurements were taken into account to obtain a better result.

According to the previous values, the determination of biobased content could be established using the following equation on the mixture powder:

$y = \delta^{13}C$ and x = Biobased % (-28.38 = a100 + b and -26.46 = b)

$\leftrightarrow$ $y = 0.0192x - 26.46$

Seven determinations have been carried out on the powder:
$\delta^{13}C$ powder: -26.66 / -26.72 /-26.76 /-26.82 /-26.82 /-26.77/-26.78‰

	$\delta^{13}C$ (‰)	Biobased %
$\delta^{13}C$ average	-26.76	15.6
$\delta^{13}C$ upper value:	-26.66	10.4
$\delta^{13}C$ lower value:	-26.82	18.7

The assessment of biobased content of this composite is evaluated at: 15% +/- 5 %

5 Conclusions

The determination of the biobased content is currently mainly undertaken using methods based on the ^{14}C methodology. In the future, in order to follow up with the growth of the bio-obased market, other methods need to be developed based on direct automation systems for ensuring regular checking of feedstocks, semi-finished components or commercial compounds.

Isotopic ratio mass spectrometry used to determine the organic isotopic ratios ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$) has been evolving since more than 70 years to become a fully automatic methodology. Originally starting on bulk mono isotopic analysis, the current available equipment proposes now multi-isotopic determinations on molecules previously separated.

Progress in instrumentation will permit in the upcoming future to develop another approach based on intra-molecular isotopic quantification.

Performances and accuracy have been similarly progressing in relation to the development of modern instrumentation.

Since about 40 years isotopic approach has been undertaken in the natural authenticity assessment, not for all components but for targeted molecules where natural and synthesis isotopic values differs significantly from each other. Thanks to the work already carried out isotopic methodology can be an alternative tool to be investigated for the bio-content evaluation. However, even if the stable isotope determination is a fully automated approach, the determination of “Direct bio-content automation” using stable isotope processes cannot be undertaken without substantial complements needed:

a) The statement of the composition of the product to be investigated, including the knowledge of the origin.
b) The isotopic reference values of the biobased components of the product and their fossil counterparts. The method will only be applicable in case of significant differences between the isotopic values from both fields of origin.

This last requirement implies to create, develop and monitor a Database for feedstocks and products.

With this in mind it will be easy to develop new methods adapted to different categories of products.

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