KBBPPS

Knowledge Based Bio-based Products' Pre-Standardization

Work package 4

Biomass Content

Deliverable N° 4.3:

Sample Preparation Techniques For Total Biomass Content Determination

Public

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1 Publishable summary

As established in the KBBPPS description of work, and reiterated on the project website (http://www.biobasedeconomy.eu/research/kbbpps/), the project aims to increase the uptake of standard test methods and certification schemes for bio-based products. It is envisiged that the development and application of standards for the bio-based product industry will have positive long-term effects on the development of a European bio-based economy. Higher quality and greater sustainability of bio-based products should increase the satisfaction of end-users at all levels, and improve the economic viability of bio-based product utilisation. Finally, public acceptance of bio-based products can be increased through ensuring and verifying the sustainable sourcing of raw materials, the effective bio-content and clear indication of their (comparative) functionality in relation to the regular products. These positive effects will result in faster growth of the bio-based product industry and increased share of bio-based in the total use of final (consumer) products and intermediates up to 2020 and beyond.

The effective determination of total bio-based content of products has been a challenge for certification bodies. The ability to determine the total bio-based content of a product is an obvious prerequisite for developing the market for bio-based products. Currently, the state of the art in terms of bio-based content determination is restricted to biogenic carbon content. The methodology is typically based on accelerated mass spectrometry to resolve the relative concentrations of carbon isotopes ($^{14}C/^{12}C$) in a sample. Results based on the ^{14}C methodology are expressed as the fraction of the bio-based carbon relative to the total carbon content of the sample. Other elements introduce a disparity between bio-carbon content and total bio-based content; some products have very few heteroatoms, such as lubricants, while more typically bio-based products such as PLA and starch composites have relatively high amounts of oxygen.

One ambition of the project is to establish a means of complete biomass content evaluation, progressing from what exists at present. Sample preparation has already been addressed in *Overview of current relevant sampling and biogenic carbon standards on global level* **KBBPPS deliverable D3.1**. Subsequent output of the KBBPPS project will provide revised radiocarbon assessment methodologies and novel research into methods for the determination of total bio-based content. The purpose of this report is the review of sample preparation techniques, bridging the gap between **KBBPPS deliverable D3.1** (which as a collection of standards designed to assist the obtaining of representative sample portions is non-specific) and actual biogenic analysis. Without any comprehensive total bio-based content determination procedures actually in place, it has had to be assumed that stable isotope analysis will be the primary tool to this effect. Accordingly the prevalence and variation of stable isotope ratios are discussed in this work, but special attention to given to the appa-

ratus used to record these ratios, for this dictates the sample preparation required. Then standard test methods for varied chemical analyses (including stable isotope analysis) that might possess attributes of interest for new standards are reviewed.

The determination of total bio-based content on the basis of molecular composition rather than isotopic abundances is also discussed. Standard test methods for various spectroscopic analyses are summarised in this portion of the report. Ultimately it is concluded that there are sufficient spectroscopy, drying, and particle size reducing procedures already established for the needs of total bio-based content determination. After these basis pretreatments isotope analysis is largely automated. What are underdeveloped (with respect to standards) are stable isotope analysis procedures, and the associated sample preparation for elements of interest for biogenic analysis.

2 Introduction

Test methods and associated calculations for bio-based carbon content are already available, but as an assessment of carbon atoms, it only accounts for a portion of the total mass of a bio-based product. To be able to measure a total bio-based content, we must be sure that the term biomass, and the products made (directly or indirectly) from it, are understood in a clearly defined way that translates to a robust and valid test methodology. The European technical committee devoted to bio-based product standardisation (CEN/TC 411) is developing definitions for biomass and products derived from biomass. A document has been produced, entitled *Bio-based products - Vocabulary*, (**prEN 16575**) and in this document is defined the term biomass and related prefixes. And so it comes to be that the following terms are defined thusly (reproduced here without adaptation for reference). The definition of bio-based elements other than carbon can be derived from the bio-based carbon definition, simply by changing carbon to the name of the required element.

- Bio-based
  Derived from biomass.

- Bio-based carbon content
  Fraction of carbon derived from biomass in a product.

- Bio-based carbon
  Biogenic carbon (carbon derived from biomass).

- Bio-based content
  Fraction of a product that is derived from biomass.

- Bio-based product
  Product wholly or partly composed of bio-based constituent(s).

- Biomass
  Material of biological origin excluding material embedded in geological formations and/or fossilized.

- Renewable material
  Material that is composed of biomass and that can be continually replenished.

The definition of biomass provided by the technical committee is a sound one by dictionary standards. Combined with the various related definitions, it implies quite logically that

the proportion of modern biological material incorporated within a bio-based product equates to the bio-based content of that article. The legacy of long deceased organisms: crude oil and inorganic fossil carbonate, do not count towards bio-based content. One extra consideration to note concerning these definitions is the use of 'bio-based content' and 'biomass content'. These two terms have been used interchangeably, but strictly speaking the former should consider materials made from a biomass feedstock and the latter relate to unmodified biomass present in a product.

Useful resources of carbon can be divided into fossil sources (crude oil and natural gas), biomass (carbohydrate, lignin, *etc*.) and inorganic carbonate minerals (limestone, chalk, and others). The mineral sources of inorganic carbon make their way into bio-based products as binders and fillers, but are routinely removed by a treatment with an acidic solution. Some solid remains, which would be calcium chloride for example if limestone had been treated with hydrochloric acid. This has no bearing on bio-based carbon analysis, but would be relevant to total bio-based content because the original product (perhaps a complex formulation) has been changed and new atoms introduced. This would not have any bearing on the total organic bio-based content.

As described in detail in the technical specification proposal by the bio-based products technical committee, entitled *Bio-based products - Determination of the bio based carbon content of products using the radiocarbon method* (**FprCEN/TS 16640**), the difference between carbon sourced from biomass and carbon sourced from fossil sources is the quantity of the ^{14}C isotope present. There is no need to elaborate further on the comments made in this technical specification, which states that "*the ^{14}C present in products is originating from recent atmospheric CO_2. Due to its radioactive decay, it is almost absent from fossil products older than 20 000 years to 30 000 years. The ^{14}C content may thus be considered as a tracer of products recently synthesized from atmospheric CO_2 and particularly of recently produced bio-products*". Accordingly, products made of a combination of bio-based materials and petroleum derived chemicals have an intermediate $^{14}C/^{12}C$ ratio indicative of the proportion of bio-based carbon in the article. The technical specification also covered sample preparation for the analysis of the ^{14}C isotope and so this will not be covered in any great detail in the subsequent chapter on isotopic analysis, except when addressed as part of combined or hyphenated analysis with other analytical techniques.

The differentiation between bio-based carbon content and carbon from other sources is well documented and forms the basis of existing standards, most notably **ASTM D6866**. A potential grey area created by inorganic carbonates is resolved by simply destroying it with acid. The bio-based carbon content cannot be taken to be equivalent to the total biomass content of a bio-based product because of the highly oxygenated chemical structure common to biomass but atypical of fossil derived chemicals. Because bio-based carbon content has already been separated from total bio-based content, then it makes sense to deconstruct bio-based chemicals completely into their constituent elements. Thus bio-based content becomes the sum of the mass contributions of each element found to be of a renewable origin

as a proportion of the entire mass of the bio-based product sample. It could be argued that the number of atoms (bio-based versus non-bio-based) could be used as the basis of determining bio-based content but mass balances are more intuitive, even if they are biased towards the larger elements. It is respected in this report that each atom is equally as important as any other in deciding the function of a compound, even though mass balance can be adopted as the primary metric. The difference between acetone and isopropanol is minor from a mass perspective but very different in terms of function and reactivity.

This report will investigate the sample preparation necessary for the analysis of bio-based products with respect to their total bio-based content. In order to do this, the method of analysis must be defined for which the sample is being prepared for. The first part of this document will describe the analysis of molecules on an elemental basis, including experimental techniques, standard test methods and sample preparation procedures, and accounts of this analysis put into practice. The second part will examine the molecules themselves that are present in bio-based products. Working at this higher level of structural complexity may help the determination of total bio-based content, without resorting to the deconstruction of the article to the more fundamental elemental level. Molecules from nature are sometimes complex beyond feasible synthetic reproduction, and exist as enantiomers. These characteristics offer the potential as a form of verification of the origin of a chemical product. The analysis required to determine whether a molecule is bio-based or not, and related standards, are also covered before the concluding remarks.

3 Total biomass content determination on the basis of elemental and isotopic analysis

3.1 Defining biomass in terms of elemental composition

The perspective that chemicals (bio-based or otherwise) are the sum of their elemental parts is useful, and as subsequently described perhaps essential, because certain characteristics of elements can be analysed in ways revealing of their origin. To try and analyse total bio-based content in any other way may prove unfeasible in practice. A certification scheme devised by ACDV (Association Chimie du Végétal, France) uses the direct analysis of bio-based carbon through radioisotope analysis and elemental analysis to verify the remaining composition [ACDV 2013]. Then the correct number of hydrogen, oxygen, and nitrogen atoms originating from biomass is decided by the supplier and a calculation of total bio-based content by mass is calculated.

Rather than allocate atoms within a molecule as either bio-derived or from fossil sources as one sees fit, direct analytical testing would remove any ambiguity. In terms of what atoms would be desirable to analyse, elemental prevalence in typical bio-based product types would need to be known. Although the analysis is only qualitative in judgement, of the total number of atoms present in bio-based products, the majority are carbon and hydrogen. This is complimented by heteroatoms of chlorine, nitrogen, and oxygen. Some examples might be poly(vinylchloride) plastic materials, ammonium salt cationic surfactants, and synthetic ester lubricants. Composite materials with inorganic binders will have increased amounts of silicon, oxygen and metal atoms contained within them. Other atoms are found in surfactants, some as counter-ions, such as sodium and chlorine, but also the sulphur of anionic surfactants. Sulphur is also a component of some solvents and additives found in lubricants.

3.2 Characteristics and origins of elements within compounds

3.2.1 Introduction to the elements

The motivation of an EU mandated drive towards a bio-based economy is the promotion of a sustainable basis of chemical product manufacture in Europe [EU M/429, EU M/492]. This is a concern because of the unsustainability of the rival petroleum chemical industry, which is the only competitor in the production of organic compounds. Only for carbon is the distinction between bio-based carbon and fossil derived carbon made unambiguous with radioisotope analysis ($^{14}C/^{12}C$ determination coupled with the ideal half-life of ^{14}C). This exact same analysis is not applicable to any other elements, not least because non of the other elements prevalent in biomass (O, H, N, S, *etc.*) posess isotopes with such ideal half-lifes. Beyond carbon, the other elements used in the synthesis of commodity chemicals and speciality formulations alike come from a variety of sources, most of them absent from fossil based hydrocarbon feedstocks. Although many require consumption of fossil resources to supply the energy needed for the processing and utilisation (*e.g.* ammonia production from N_2 and H_2).

The example of the surfactant sodium laureth sulphate demonstrates that the atoms that comprise the product are sourced from petroleum, biomass, gases condensed from the air, water, and mineral sources (**Figure 3.1**). The implications of these five sources of elements for chemical manufacture should be contemplated in more detail, for it reiterates the important distinction between organic and inorganic content and may confuse the assessment of bio-based content. Only what is considered as the organic content of bio-based products is assessed in current bio-based carbon content assessments, all of which are based on **ASTM D6866**. Eliminating the inorganic component in total bio-based content analysis as well could be used to essentially disregard elements from mineral sources. This would reduce the number of elements that need to be considered and simplify the assessment somewhat.

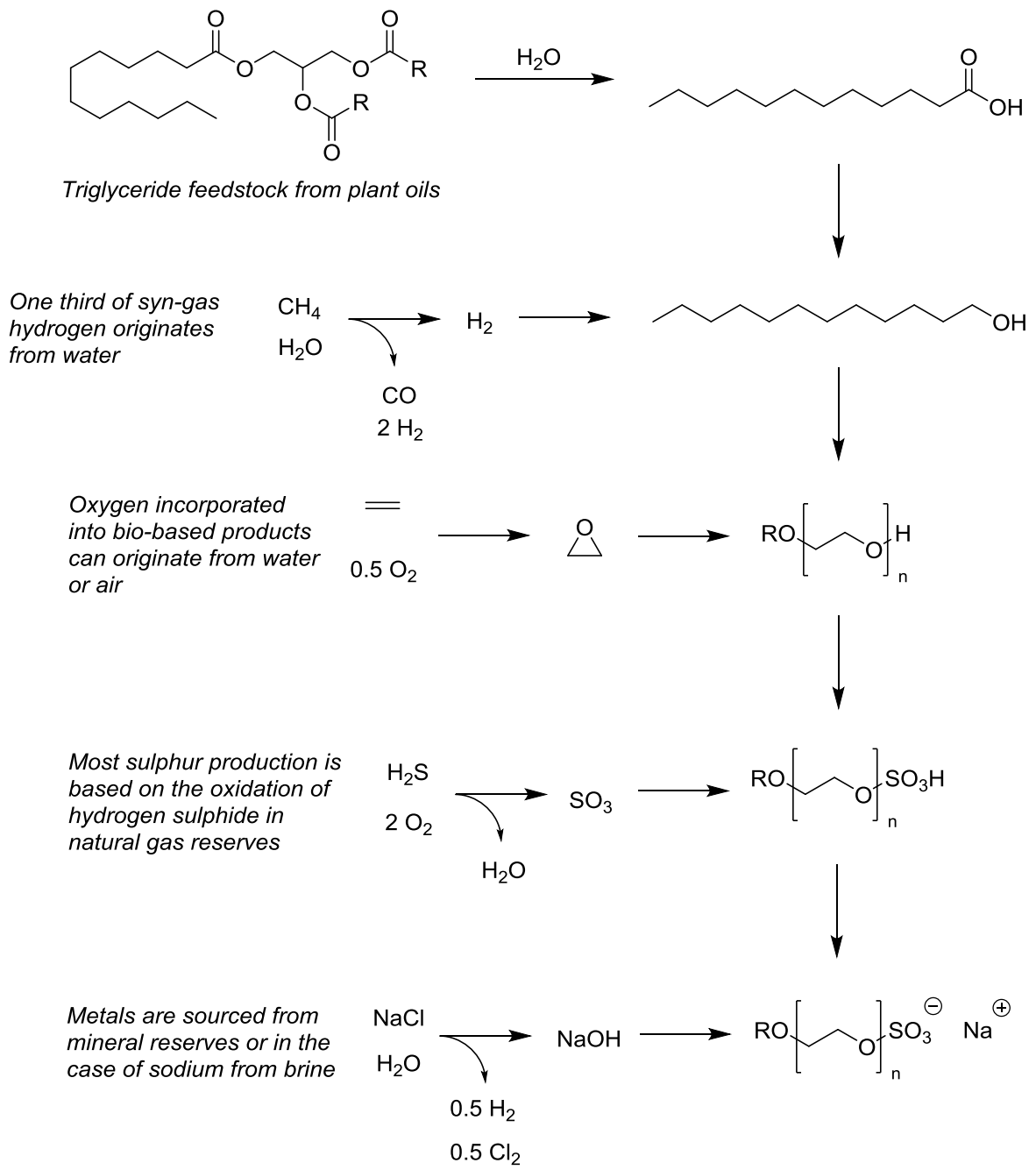

Figure 3.1 The synthesis of a surfactant from a variety of sources.

If by claiming 44 wt% of the mass of the total atoms in sodium laureth sulphate (possessing a trimer PEG chain moiety, n = 3) are bio-based, the implication is that 56% of the mass of the compound is fossil derived. If an equally correct claim is made that 28 wt% of the product by mass is fossil derived, then a logical assumption might be that 72% of the sodium laureth sulphate is bio-based (**Table 3.1**). The discrepancy comes from atoms introduced from water as a feedstock, oxygen condensed from the air, and mineral sodium chloride. For this example it is assumed that the sulphur content originated from hydrogen sulphide in natural gas, and hydrogen gas was sourced from syn-gas production without an additional wa-

ter-gas shift reaction. It should be made very clear whether it is bio-based content that is desirable to know, or non-fossil derived content that should be reported. Elements originating in water and air might be assumed as equivalent to biomass and incorporated into total biomass content values because they are relatively benign and not unsustainable as petroleum is. Mineral sources, although many are much scarcer and more vulnerable to depletion than petroleum, could be allied with bio-based content for convenience. Alternatively total bio-based content could be decided on the basis of organic content and most of the elements ignored. This would be difficult for surfactants featuring a mineral sourced counter-ion. Regardless of the exact approach taken, the manner in which total bio-based content is calculated should be disclosed, but first chosen with careful consideration.

Table 3.1 The origins of elements present in sodium laureth sulphate (n = 3) and their mass contribution to the molecule.

	C	H	O	S	Na	Total
Fossil	17%	3%	0%	8%	0%	28%
Bio-based	34%	6%	4%	0%	0%	44%
Water	0%	0.16%	0%	0%	0%	<1%
Air	0%	0%	23%	0%	0%	23%
Mineral	0%	0%	0%	0%	5%	5%
Sum	51%	9%	27%	8%	5%	100%

The sodium laureth sulphate case study was constructed from tracing the elements from their supposed feedstocks through proposed manufacturing processes. In reality this will be extremely difficult to put into practice for complex mixtures that are formulated by chemical suppliers late in the supply chain. More of this discussion will form the basis of a subsequent report (**KBBPPS deliverable D4.5**, to be published in 2015). Instead, a characteristic of each element dependant on its source could be manipulated for a direct biomass content analysis. This can be coupled with an elemental analysis to determine the amount of each element in the bio-based product. Stable isotopic ratio analysis may become useful in this regard. The ratios of naturally occurring and non-decaying isotopes that exist for many elements differ under natural influences, making the identification of their geographical origin possible. Examples of potentially useful isotopes present in nature are presented in the following table (**Table 3.2**) [Ehleringer 1989].

Table 3.2 Isotopic abundances of elements of ecological interest.

Element	Isotope mass	Abundance
Hydrogen (H)	1	99.985%
	2	0.015%
Carbon (C)	12	98.89%
	13	1.11%
Nitrogen (N)	14	99.63%
	15	0.37%
Oxygen (O)	16	99.76%
	17	0.04%
	18	0.20%
Magnesium (Mg)	24	78.70%
	25	10.13%
	26	11.17%
Silicon (Si)	28	92.21%
	29	4.70%
	30	3.09%
Sulphur (S)	32	95.00%
	33	0.76%
	34	4.22%
	36	0.014%
Chlorine (Cl)	35	75.53%
	37	24.47%
Potassium (K)	39	93.10%
	40	0.0118%
	41	6.88%
Calcium (Ca)	40	96.97%
	42	0.64%
	43	0.15%
	44	2.06%
	46	0.0033%
	48	0.18%
Iron (Fe)	54	5.82%
	56	91.66%
	57	2.19%
	58	0.33%
Copper (Cu)	63	69.09%
	65	30.91%
Zinc (Zn)	64	48.89%
	66	27.81%
	67	4.11%
	68	18.57%
	70	0.62%

3.2.2 Carbon isotopes and origins

Even if ^{14}C isotope analysis remains the cornerstone of analytical bio-based content determination methods, stable isotope ratios may act as at least a supplementary means of providing further information about the other elements. Because of the definitive nature of carbon radioisotope analysis, it might be perceived that $^{13}C/^{12}C$ ratios are somewhat redundant. It is true that, as for other elements, sometimes broad ranges of stable isotope ratios rather than precise values define the possible origin of that element. However the combination of stable isotope analyses belonging to different elements (including carbon) increases the precision of this method, and is addressed accordingly in due course.

The two natural stable isotopes of carbon, ^{12}C and ^{13}C, have become unequally distributed throughout the biosphere. The atmospheric proportion of ^{13}C resides within a narrow range of variation (1.101% to 1.105% of total carbon atoms) [Francey 1999]. The uneven distribution has its origins in different types of plant metabolism disfavouring the heavier isotopes of carbon to different degrees. The following equation (**Equation 3.1**) is necessary to calculate the isotopic composition of carbon, but is directly applicable to other elements as well:

Equation 3.1

$$\delta^{13}C\ (‰) = \left[\left(\frac{^{13}C_{sample}}{^{12}C_{sample}} \right) \Big/ \left(\frac{^{13}C_{standard}}{^{12}C_{standard}} \right) - 1 \right] \cdot 1000$$

A study on the bio-based content of plastics uses stable carbon isotopic ratio analysis [Suzuki 2010]. What the Japanese research team discovered is that the known propensity of plants to disfavour the ^{13}C isotope depending on their carbon fixation process (C_3 or C_4 type metabolism) manifests itself in downstream plastic products made from sugar fermentation products and starch [Suzuki 2010]. This is of course not unexpected but a pre-requisite for any meaningful stable isotope analysis in the field of bio-based product certification.

Chemicals made from a petroleum feedstock have a carbon isotopic composition equivalent to rice, wheat, and potato. Ancient plants operated the C_3 carbon fixation process, which is preserved in the three aforementioned plant species. Corn and sugarcane, two popular bio-chemical feedstocks, have evolved a different carbon fixation process that disfavours ^{13}C to a greater extent (C_4 metabolism). Thus corn and sugarcane carbohydrates and the plastics made from them, by fermentation or direct application, are distinguishable from rice, wheat, potato, and fossil derived chemicals (**Table 3.3**). In the sample set, it was shown that polyethylene (PE) made from sugarcane was distinguishable from that made from petroleum derived ethene. In addition, polylactic acid (PLA) made from corn was also inconsistent with a chemical of a non-renewable origin, but its $\delta^{13}C$ is not discernible from that of carbonate

minerals. Obviously PLA made from wheat starch would not be clearly distinguishable from a petroleum derived equivalent using this method, highlighting the major limitation of this technique when used in isolation.

Table 3.3 Carbon isotopic composition ranges for samples of material from different feedstocks [Meier-Augenstein 1999, Suzuki 2010].

Feedstock	Fixation mode	Minimum $\delta^{13}C$	Maximum $\delta^{13}C$
Carbonates	n/a	-14‰	+4‰
Corn	C_4	-10.8‰	-10.7‰
Petroluem	n/a	-32.5‰	-23.3‰
Potato	C_3	-27.2‰	-25.8‰
Rice	C_3	-27.5‰	-26.6‰
Sugarcane	C_4	-15.1‰	-12.1‰
Wheat	C_3	-27.7‰	-27.6‰

Analysis of a formulated product or composite would not be able to determine the precise ratio of plant derived to non-renewable material, even if only corn or sugarcane was used as a source of biomass, because the range of data scatter is large. The regions that C_3 and C_4 products occupy do not overlap with each other, but because of the possible range of $\delta^{13}C$ values attributed to each feedstock, mixed source articles cannot have a biomass content associated to them with any reliability. Take the example of a composite material made from an equal mass of sugarcane derived PE and traditional petroleum derived PE (**Figure 3.2**). The experimental ^{13}C isotopic analysis could provide a carbon isotopic composition of anything between -23.8‰ and -17.7‰. The lower end of this range overlaps with the domain covered by crude oil, and so with this analysis it could be concluded that the article is 0% bio-based. Conversely if the best possible -17.7‰ ^{13}C isotopic composition was observed then this could represent anything up to 85% bio-carbon content. Obviously this possible range of answers is far from satisfactory.

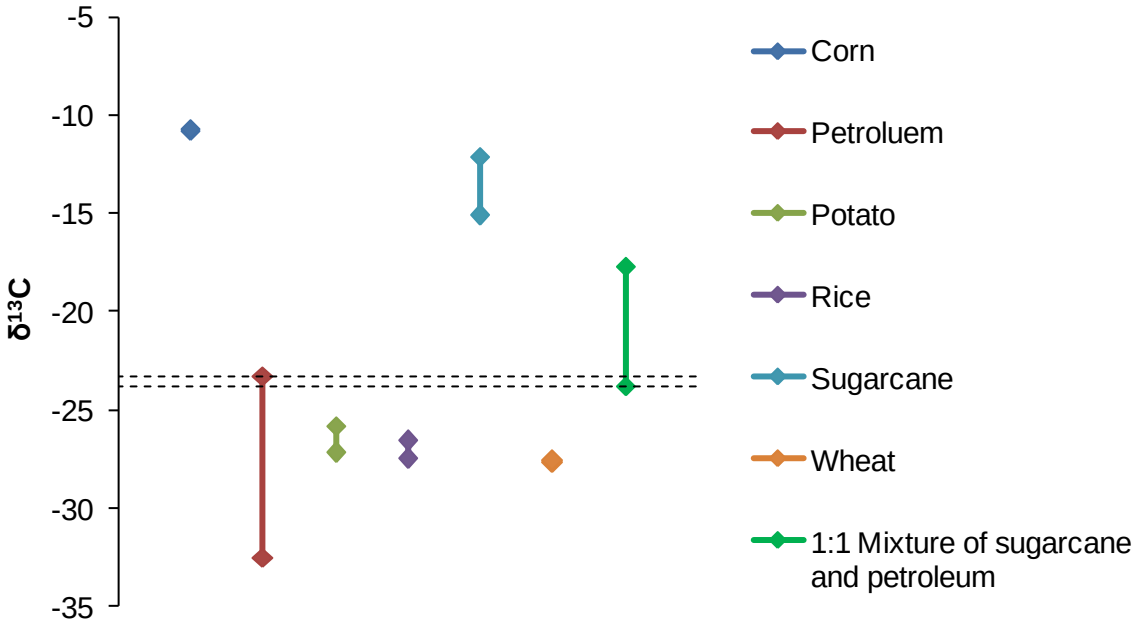

Figure 3.2 A hypothetical sugarcane-petroleum derived composite characterised by ^{13}C isotopic analysis. The overlap in possible results of fossil based material and a supposedly 50% sugarcane derived material are indicated by the dashed lines.

3.2.3 Hydrogen isotopes and origins

Hydrogen is the other obvious elemental component of crude oil and natural gas reserves in addition to carbon. For making the distinction between biomass derived products and those made from fossil based feedstocks the analysis of carbon and hydrogen is most important. Sulphur is the only other element that is acquired from fossil feedstocks, in the form of the gas hydrogen sulphide, but it is less common as an element in bio-based lubricant, plastic, and solvent products, each with large markets. An obvious argument against placing hydrogen on a pedestal alongside carbon is its relative atomic mass. Mass contributions will be used as the basis of bio-based content, but neither is that an excuse to simply dismiss the lighter elements. Each element is equally essential to the molecular structure and function of a molecule.

There are three well known isotopes of hydrogen, so much so that each isotope has their own name: protium (^{1}H), deuterium (^{2}H), and tritium (^{3}H). Tritium has a half life of a little over 12 years, but is constantly renewed in the atmosphere when a neutron interacts with a nitrogen atom, meaning that biomass contains a near maximal proportion of tritrium although this is only a tiny amount (les than 10^{17} protium atoms for every tritium atom in the atmosphere) [Neary 1997]. This provides an obvious handle for the differentiation between recent biomass samples and bio-based products derived from biomass, and petroleum derived chemicals. Analysis has shown bio-based benzaldehyde can be distinguished from fossil based benzaldehyde on the basis of tritium content [Neary 1997].

More than one in every 7000 hydrogen atoms found in the oceans is deuterium, a much higher concentration than tritium enjoys amongst hydrogen atoms [Gonfiantini 1978]. Accordingly deuterium might be a better choice of nucleus to form the basis of a biogenic analysis, although we are yet to address the techniques available for doing so and the associated sample preparation. It has been found for methane samples that ancient natural gas and methane trapped in coal is differentiable from modern, naturally occurring sources of biogas through the abundance of deuterium observed (**Figure 3.3**). Methane produced from the inefficient burning of biomass shows some overlap between itself and the relative deuterium content of petroleum sources [Snover 2000]. Similarly fermentation products (demonstrated here with acetic acid in vinegar) express a range of δ^2H values, also overlapping with fossil based hydrogen atoms [Hattori 2010]. The difference in δ^2H data ranges created by different natural processes acting on biomass (anaerobic digestion, biomass burning, or fermentation) is an additional variable to consider when attempting to translate stable isotope ratios into claims of biogenic origin.

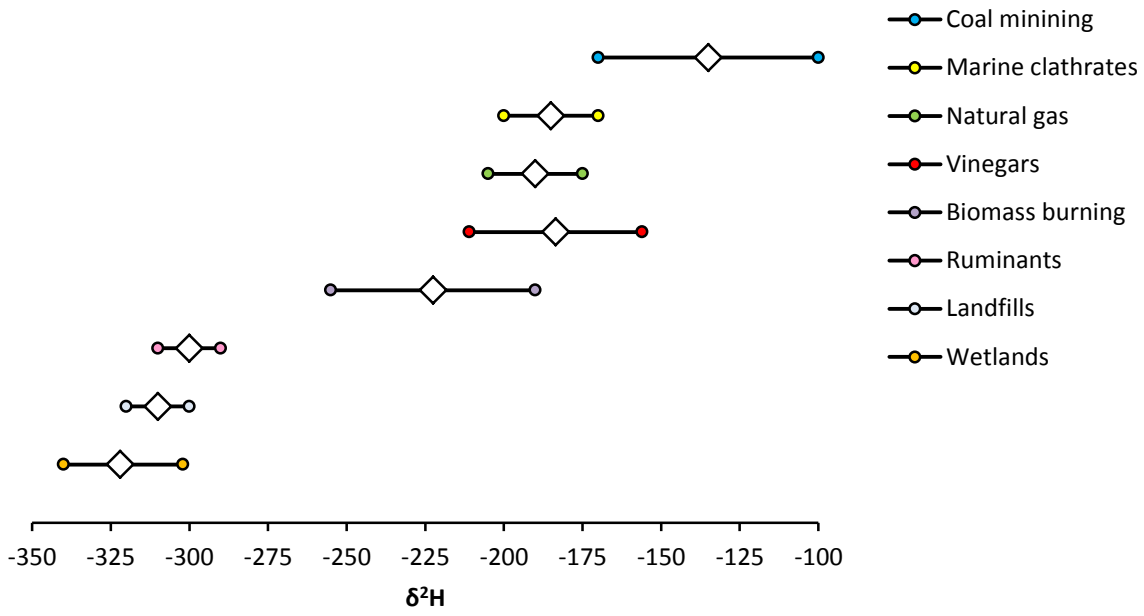

Figure 3.3 A reinterpretation of literature δ^2H data from different methane sources [Hattori 2010, Sowers 2006].

3.2.4 Chlorine isotopes and origins

Chlorine has two major stable isotopes, with the lighter isotope, ^{35}Cl, present in an approximate 3:1 excess over the heavier ^{37}Cl isotope [Ehleringer 1989]. In the absence of a significant natural isotopic fractionation process, the relative ratio of these two isotopes is constant, and therefore not of any use in biogenic analysis. Another isotope of chlorine exists, but ^{36}Cl is radioactive. So in the same way that $^{14}C/^{12}C$ isotopic ratios provide a good

indicator of the modern bio-based content of a sample, $^{37}Cl/^{36}Cl$ may be able to do the same. The half-life of ^{36}Cl is approximately 300,000 years [Elmore 1987]. Only the lack of chlorine atoms in biomass and petroleum feedstocks undermines the utility of this potential methodology.

The production of chlorinated compounds is dependent on the electrolysis of brine to give chlorine gas. Organohalides (chloroform, poly(vinyl chloride), *etc*.) are made by the reaction of chlorine gas, or sometimes bleach or hydrogen chloride, with both saturated and unsaturated hydrocarbons [Weisermel 1993]. Inorganic chlorides will have a direct mineral source in most cases. This being the case, the need for stable isotope analysis of chlorine for the purpose of bio-based content analysis may be redundant.

One application of chlorine radioisotope analysis is chlorinated solvent monitoring in groundwater. Bio-based solvents are unlikely to contain chlorine given recent legislation such as REACH [EC 1907/2006], but chlorine should be addressed more broadly because it occurs in cationic surfactants and the inorganic components of formulations. It has been found that $^{37}Cl/^{36}Cl$ ratios are not consistent across batches of chlorinated solvents, varying according to supplier and year of production [Beneteau 1999]. Because of this observation, and the lack of diversity in sources of chlorine, it is difficult to have confidence in this analysis as a means of establishing a measure of bio-based chlorine content.

3.2.5 Nitrogen isotopes and origins

Nitrogen is incorporated into biomass, most notably as an elemental component of proteins. Synthetic, non-biological sources of nitrogen originate exclusively from ammonia, which in turn is produced by the Haber-Bosch process that combines the gases of nitrogen and hydrogen. The dual fossil-water source of hydrogen gas was briefly addressed as part of the previous sodium laureth sulphate case study (**Figure 3.1**). Nitrogen is condensed from the air, a massively abundant source of nitrogen established billions of years ago, and also utilised by nitrogen fixing plants as their source of nitrogen.

Nitrogen is found in nature predominately as the ^{14}N isotope, with only 0.37% ^{15}N observed; both are stable isotopes [Ehleringer 1989]. Due to metabolism pathways acting faster on lighter isotopes of nitrogen, $^{15}N/^{14}N$ ratios increase up the food chain. Similarly, nitrates in soil also tend to have enhanced $\delta^{15}N$ values because of microbial action. Nitrogen fixing has no such bias [Ehleringer 1989]. This means that legume plants obtaining nitrogen from the air will have a different ratio of nitrogen isotopes than that of plants obtaining nitrogen from minerals in the soil. The use of a fertiliser will also directly impact the $\delta^{15}N$ value of the plant matter. Nitrate fertilisers are synthesised from atmospheric nitrogen *via* the Haber-Bosch then Ostwald processes [Ostwald 1902, Ostwald 1903].

3.2.6 Oxygen isotopes and origins

Oxygen is one of the three most abundant elements in biomass, evidence of which can be seen in the prevalent bio-polymers cellulose and lignin. Plants access oxygen through the process of photosynthesis, using carbon dioxide and water as their feedstocks, although the majority of the atomic oxygen content of the two reactants is ejected as waste. The carbon cycle is completed, at least in part, by respiration of organisms that use that waste oxygen and replenish the carbon dioxide and water needed by the plants to produce more valuable biomass. The death and fossilisation of organisms that in the past lead to the formation of crude oil and natural gas resulted in biomass being reduced to hydrocarbons. Oxygen makes up a tiny proportion of the mass of fossil fuel reserves, with a similarly insignificant amount associated to nitrogen and most other elements [Sergeant 1995].

As is done for nitrogen, oxygen is obtained from the air for a number of industrial chemical manufacturing processes. Direct oxidation with molecular oxygen is common; the synthesis of aldehydes from alkenes is one example. Sometimes oxygen is used to make dehydrogenation more energetically favourable without actually being incorporated into the final product, as in the synthesis of hydrogen cyanide from methane and ammonia [Weissermel 1993]. Water is another source of oxygen in the synthesis of compounds. Ethanol is made by the hydration of ethylene. Ethylene glycol is made from the hydration of ethylene oxide, in turn the product of the reaction between ethylene and oxygen (**Figure 3.4**).

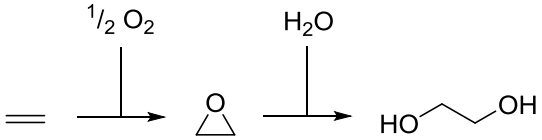

Figure 3.4 The use of molecular oxygen and water as sources of oxygen in the synthesis of ethylene glycol.

The isotopic ratio of oxygen samples is not directly dependant on the age of the sample as it is with $\delta^{14}C$. Ice core samples use $^{18}O/^{16}O$ stable isotope ratios to indicate global temperatures of the past. Semi-empirical equations of the following type are used to model historical temperatures (**Equation 3.2**) [McCrea 1950]:

Equation 3.2 $$\delta^{18}O\ (‰) = \frac{16200}{T\ /K} - 56.8$$

Because the water cycle is dependent on evaporation, and this is affected by the extra mass of $H_2{}^{18}O$ (compared to $H_2{}^{16}O$) and the ambient temperature, it is not viable to use oxygen isotopic ratios as the sole basis of an indicator of geographic location or age, and by inference biogenic origin.

3.2.7 Silicon isotopes and origins

Plants discriminate between the stable isotopes of silicon as they do with carbon isotopes and others. Generally $^{30}Si/^{28}Si$ ratios observed in plants favour the lighter isotope relative to the neighbouring soil. The $\delta^{30}Si$ values found in the actual soil silicates vary too, and so the combination of these variables makes absolute tracing of silicon more difficult [Opfergelt 2008].

Recently silica has begun to be retrieved from the ashes of burnt biomass as a source of bio-based silicon [Dodson 2013]. Its origin is the minerals incorporated in soil based nutrients, but unlike phosphorus for example, which is taken up by plants in the same way as silicon, the process for reclaiming silicates is being developed for commercial purposes. Therefore a distinction has been established between mineral reserves of silica and plant waste sources. The actual difference between inorganic mineral silica, and silica that has resided within a plant for a short period of time, and its implications, should be considered. The definitions adopted for bio-based products will direct this, and as an increased number of products containing bio-based silica are produced, more thorough analysis can be performed to resolve this issue.

3.2.8 Sulphur isotopes and origins

The sulphur cycle is complex, and involves intricacies such as volcanic eruptions, but the decomposition of biological organisms is the major force in sulphur transfer [Thode 1991]. Sulphur is produced from mineral sources, but current production trends are establishing a dominance of petroleum derived sulphur [Bixby 1983]. Thus sulphur is the third and final element obtained from fossil sources to consider, accompanying carbon and hydrogen. This should heighten attention on sulphur as an element of importance when determining the bio-based content of chemical products.

The hydrogen sulphide in natural gas, sometimes a considerable proportion of the reserve, is oxidised to sulphur with the Claus process [Nehb 1985]. Usually it is oxidised further to sulphur trioxide to make sulphuric acid. Sulphur is often found in anionic surfactants but not in most other bio-based product classes. The sodium laureth sulphate example given previously is synthesised through the reaction between sulphur trioxide and an alcohol (**Figure 3.1**). Some lubricant additives are sulphur based but these are not likely to be classifiable bio-based compounds.

Differentiation between ^{34}S and ^{32}S has been performed on meats and alcoholic beverages [Kelly 2002]. It was found that for the data set as a whole, an overlap occurred between mineral sulphur reference salts and fossil sources of sulphur with bio-based foodstuffs. An additional concern is that it seems from this study that the sample preparation influences the result. This will be covered in *section 3.5* in greater detail. Pollution has long

been known to also increase $\delta^{34}S$ values [Ehleringer 1978, Winner 1978]. The natural creation of crude oil is not thought to influence stable isotopes of sulphur by isotopic fractionation, and therefore the $\delta^{34}S$ range of plants (approximately -30‰ to + 30‰) is fairly well persevered in fossil based sulphur (**Figure 3.5**) [Ehleringer 1978, Hirner 1989]. Therefore the quite unfortunate conclusion that must be reached is that the three feasibly petroleum derivable atoms (sulphur, along with carbon and hydrogen) do not possess absolutely distinct isotopic ratios required to determine total bio-based content without ambiguity.

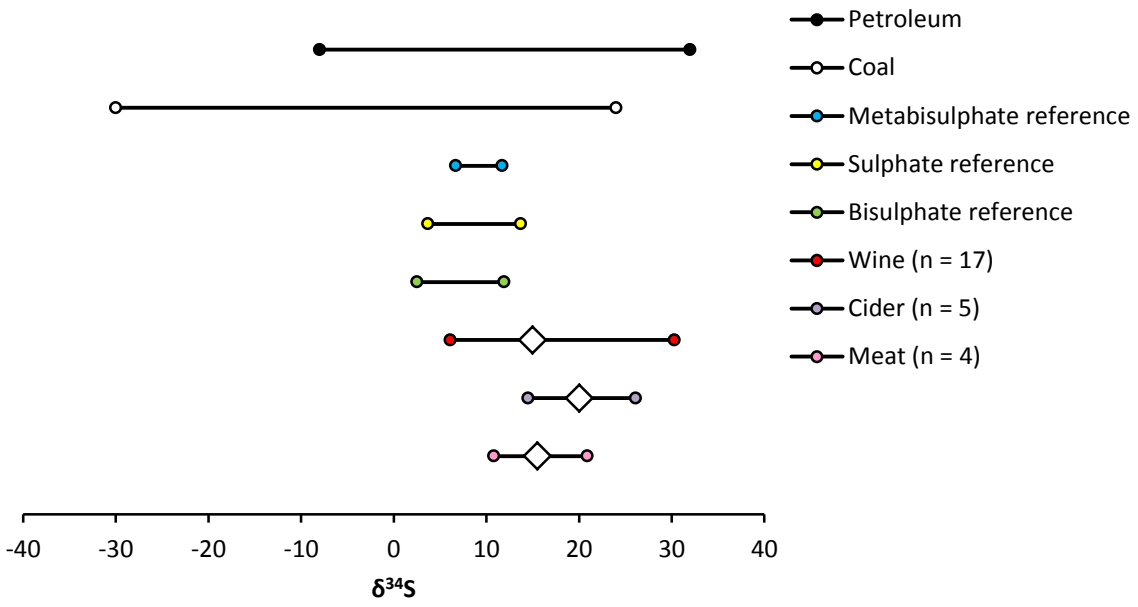

Figure 3.5 Sulphur isotopic abundance ranges and for food products data mean averages [Kelly 2002, Thode 1991].

3.2.9 Complete bio-based isotopic analysis

Many elements do not have more than one stable isotope and so are not compatible with this sort of analysis. Phosphorus only has one stable isotope (^{31}P), and so inferring the origin of a phosphorus atom cannot be done by direct methods of isotope ratio analysis. However the source of phosphates can be traced using the isotopic signature of bonded oxygen [Paytan 2011]. Because $\delta^{18}O$ is determined by temperature and not location or age, this analysis is not precisely indicative of whether the phosphate is obtained from mineral sources or from biomass. Phosphorus is present in biomass; it is quite abundant in willow compared to most other elements [Raunemaa 1986]. In many ways phosphorus reserves should be managed with a greater cautiousness than biomass feedstocks [Cordell 2009, Vuuren 2010] However, unlike silicon for example, bio-based phosphorus is not an economically viable source of phosphorus, which curtails the usefulness of a biogenic analysis on this

element. Instead phosphorus is mined in the form of calcium phosphate, and usually processed *via* phosphoric acid into higher values chemicals.

Sodium, of which ^{23}Na is the only stable isomer, is one of several metal elements that regularly form a component within different bio-based products. Sodium metal and its compounds are produced from sodium chloride [Klemm 1985]. Sodium carbonate is the only other commercially attractive source of sodium, but this too is often synthesised from sodium chloride [Lemke 1983]. Its role as a counter-ion means it is not a prominent component (by function) in any material or formulation of interest, but it is present nevertheless. If the sodium portion of sodium laureth sulphate was to be considered as inorganic content, a process could be developed to remove it, as inorganic carbonates are, but at the expense of dramatically modifying the article. It is unlikely that metal elements will be considered as a contributor to the total bio-based content of a bio-based product in most cases.

The discussion of stable isotope ratios (and the elements without more than one stable isotope) has highlighted that the definition of the term bio-based, and the initiative to enhance the bio-based economy, has established a distinction between biomass sources of chemicals and other sources of the elements, presumed to be fossil based and undesirable. Both EU mandates do not mention fossil feedstocks, but recall a statement of the EC competitiveness council, calling for the "*fostering emergence of markets with high economic and societal value"*, and then give the rationale for a bio-based economy on the basis of jobs, sales, and monetary value [EU M/429].

Only carbon, hydrogen, and sulphur are obtained from petroleum. Biomass feedstocks, although rich in carbon and hydrogen, are generally not a viable source of sulphur (a possible exception in the future being phytoplankton emissions of dimethyl sulphide) [Charlson 1987]. Other elemental reserves consist of the air, sea water, and mineral deposits. There are severe sustainability concerns over the future of many mineral reserves but this is out of the scope of this report and the KBBPPS project as a whole. Analysis of all elements with measureable stable isotope ratios will not be discounted because this issue has not been resolved, but a strong emphasis on carbon, hydrogen, and sulphur should be established as only for these elements is there competition between petroleum and biomass feedstocks.

3.3 Elemental and isotopic analysis techniques

3.3.1 Elemental analysis

Routine elemental analysis is of the type that returns data on the mass contribution of carbon, hydrogen and nitrogen atoms contained within the sample (CHN analysis) [University of York 2013]. Oxygen content is performed separately, for this method of CHN analysis uses excess oxygen to combust the sample. Other elements can also be detected such as sulphur and chlorine using less conventional apparatus, but usually simultaneous detection of elements (at least carbon, hydrogen, and nitrogen) is used [Kirsten 1983].

Sample preparation for elemental analysis will be covered again in subsequent discussions of isotope analysis. For here it is useful to know that the sample, perhaps only 2 mg of material, is placed in a tin vessel and sealed. The sample should be representative of the whole product being tested, adhering to proper sampling techniques described in a previous report of the KBBPPS project (**KBBPPS deliverable D3.1**). From this point on, the process may be automatized (including auto-sampling) so that samples can be queued on the system.

Inside the elemental analyser, temperatures exceeding 1250 K in an oxygen enriched atmosphere combusts the sample [University of York, 2013]. To ensure complete combustion, a tungsten oxide catalyst is present to yield carbon dioxide, water, and nitrogen oxides deriving from the sample. A reducing oven consisting of a column packed with copper at 893 K reduces nitrogen oxides to dinitrogen gas. So that only carbon dioxide, water, and nitrogen proceed onto the chromatographic stage, chemical traps that remove corrosive sulphurous oxides and HCl are required. This signals the end of sample preparation.

To obtain results corresponding to the amounts of carbon, hydrogen, and nitrogen in the sample, gas chromatography is performed on the mixture of combustion products. The detection of the gases can be performed by detecting changes in thermal conductivity compared to the carrier gas (helium), proportional to the amount of the component present [Kirsten 1983]. The absolute quantity of each gas is measured but not reported as such. Instead values are calibrated against a reliable reference material. This process is not suitable for the detection of sulphur oxides, and so photoionisation is used instead. The sensitivity of the apparatus when assessing a sample of 2 mg is sufficient enough to result in an error of only 0.3 wt% in most cases.

For the determination of oxygen up to 5 mg of sample is required, but a silver vessel is used and not the more routine tin capsule. A helium atmosphere replaces the more usual oxygen atmosphere for obvious reasons. The oxygen present in the sample is converted to carbon monoxide through a reaction with a nickel-carbon composite [Preston 1992]. Gas chromatography is required to separate CO from nitrogen gas which may be evolved de-

pending on the composition of the sample. The separation of these two gases is important because and the consequence of not chromatographically resolving the nitrogen will lead to an over-estimation of oxygen content. Detection is once again by thermal conductivity measurements.

This methodology for obtaining elemental analysis is perfectly acceptable for solids, but trickier for liquids and not applicable to gases. Other issues relate to the presence of phosphorus and fluorine [University of Wien 2013]. Phosphorus inhibits the required combustion of the sample, forming minerals containing carbon, hydrogen, and oxygen instead. Fluorine will react under the conditions of the combustion oven to give hydrogen fluoride which in turn reacts with the silica packed into the columns. The analysis of oxygen is also affected by fluorine, which through the reaction of hydrogen fluoride with silica liberates water, in turn reacting with carbon to give carbon monoxide in the hot helium atmosphere of the elemental analyser. Obviously this will create a false positive error as carbon monoxide is the molecule from which the oxygen content of the original sample is calculated.

A certification scheme developed by ACDV, the French *Association Chimie du Végétal*, supplements direct ^{14}C isotope measurements of bio-based carbon (**ASTM D6866**) in the characterisation of bio-based products with an elemental analysis [ACDV 2013]. The elemental composition of a sample does not give any direct evidence towards the origin of those elements within the sample. So for single (pure) compounds, the elemental analysis is accompanied by a declaration by the chemical supplier suggesting the origin of those elements (either bio-based or fossil derived, no option for mineral, water, or air sources is permitted). Obviously such a declaration is wholly dependent on the interpretation of the term bio-based adopted by the supplier, and the information available to them, to compliment what information is gained from bio-based carbon content analysis and elemental analysis. If the suggested total bio-based content of the product is consistent with elemental analysis and ^{14}C abundance then a certificate is delivered. The organisation request 100 g of sample, with brief instructions requested to help ACDV with their sample preparation, which consists only of sample size reduction. Many standards address sample size reduction, some of which are reviewed later within this document.

The example ACDV use themselves is the hypothetical molecule ethyl acetate made from bio-ethanol and fossil based acetic acid (**Figure 3.6**). From the elemental analysis the supplier then allocates the proportion of each element originating from biomass and the remainder attributed to a petroleum source (**Table 3.4**). Given the only definitive analysis of elemental origin within this scheme is ^{14}C isotopic analysis, it makes sense to attribute the heteroatoms directly bonded to fossil based starting materials as petroleum derived in the final product. Similarly, the exercise is simplified if non-carbon atoms directly bonded to bio-based carbon are regarded as bio-based also. Of course the expected result of 50% bio-based carbon content does not confirm that the two carbon atoms of the ethyl moiety are the bio-based carbon atoms. Once again this is inferred from the declaration of the supplier.

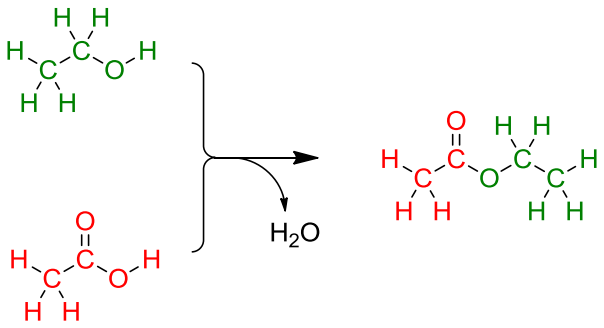

Figure 3.6 The synthesis of ethyl acetate from bio-ethanol (red atoms are assumed to originate from fossil sources, green atoms are assumed to be from biomass).

Table 3.4 The ACDV allocation of atomic origins for a partially bio-based ethyl acetate molecule.

Source	C	H	Other	Total
Bio-based	27.3%	5.7%	18.2%	51.2%
Fossil based	27.3%	3.4%	18.2%	48.9%
Total	54.5%	9.1%	36.4%	100%

In order to keep the calculation simple some errors are introduced. For example, there is no oxygen in fossil sources of chemicals, and so regardless of whether the oxygen content is derived from biomass, water, or the atmosphere, attributing it to the fossil based content of the sample is incorrect. The question then becomes a choice between allying all non-petroleum sources of oxygen with biomass or creating a number of alternative categories. This is covered in a following report (**KBBPPS deliverable 4.5**). Some concern should be expressed over the assignment of this ethyl acetate compound as 51% bio-based, when it could be up to 70% in real terms (**Figure 3.7**). Thus inferring the total bio-based content by extrapolating it from elemental analysis, justified only by an understanding of the synthesis of that molecule, may not resolve the limitations of direct bio-based carbon content determination as it currently stands. It is true that the elemental analysis creates a good basis for further investigations, especially stable isotope analysis, and although this was overlooked by ACDV, it is discussed in this report.

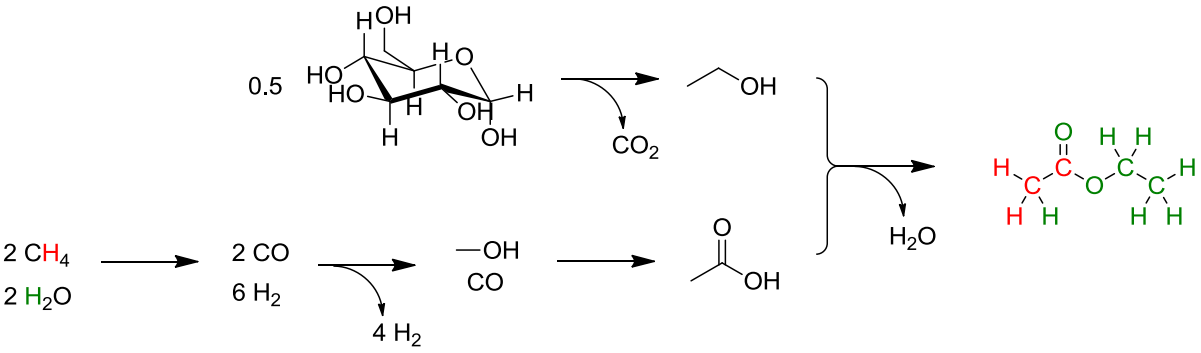

Figure 3.7 An accurate allocation of the elemental origins of partially bio-based ethyl acetate.

Formulations are also addressed by this ACDV methodology, but unless their substituent parts have been certified separately as individual pure compounds the ingredients are considered to be non-renewable. The mass contributions and the total bio-based content of each ingredient (when known) are multiplied to give a value of bio-based content for the formulation. Carbon radioisotope analysis is equally applicable to mixtures as it is to pure compounds and this part of the certification process remains.

Another certification scheme, this time run by Vinçotte and entitled 'OK-Biobased' is also used to certify bio-based products [Vinçotte 2013]. However the procedure only requires a total organic carbon calculation and a measurement of $\delta^{14}C$ for bio-based carbon content, *à la* **ASTM D6866**. Although unambiguous, it is similarly limited as the US 'BioPreferred' programme, which labels the product and its packaging with a percentage bio-carbon content and no more [USDA 2013].

3.3.2 Elemental mass spectrometry

Mass spectrometry (MS) is often used in combination with CHN elemental analysis, or on its own in preference to elemental analysis, for the structural elucidation of organic compounds. The principle behind mass spectrometry is that the ionisation of the sample, followed by interaction with an electromagnetic field, will separate species on the basis of charge and mass. But the information gained from the mass spectrometry of molecules is exactly that, an analysis of molecules and any associated molecular fragments. The next chapter covers spectroscopy and spectrometry of bio-based molecules more broadly.

Mass spectrometry can be used as a method of detection, hyphenated to inductively coupled plasma (ICP) ionisation apparatus. Now atoms are analysed rather than their compounds. A sample (solids are dissolved in a solvent) is introduced to argon plasma at a localised temperature of 10,000 K as a nebulised aerosol mist, or by laser ablation of a solid directly [Russo 2002]. The sample is atomised by ICP, and cations formed of the individual atoms. Mass spectrometry can then be conducted on the atoms for an analysis of the elemental composition of the sample. Most atoms can be detected by ICP-MS, but most atoms are metals not applicable to the majority of bio-based products. Because the organic matter in the sample is incinerated in the ICP process, the key elements of carbon, hydrogen, oxygen, nitrogen, *etc*. are not detectable. Atomic emission spectroscopy (AES) can also be used as a detection method. The technique of ICP-AES is usually applied to metals too, and so also has less importance with respect to bio-based products.

3.3.3 Isotopic mass spectrometry

Usually the only indication of the presence of isotopes in routine mass spectrometry comes from the marginally significant occurrence of ^{13}C (approximately 1% natural abun-

dance), and then this is only clear in larger molecules. However, the most sensitive techniques (four orders of magnitude more sensitive than typical MS) are able to resolve a greater number of isotope ratios in simple compounds [Blessing 2008]. Accelerator mass spectrometry (AMS) is achieved at the expense of flexibility in what can be detected [Brenna 1997, Meier-Augenstein 1999]. Suitable substrates for isotopic ratio mass spectrometry (IRMS) are typically obtained by combusting a sample to give, for example, carbon dioxide for carbon isotope analysis. The combustion can be performed by an elemental analyser (EA), where the sample is typically oxidised at temperatures of above 1000 K with chromium and cobalt oxide catalysts to give carbon dioxide and sulphur dioxide, then passed through a reductive furnace packed with copper to produce nitrogen gas from any nitrous oxides also in the sample gases. Although water is the chemical basis of hydrogen elemental analysis, hydrogen gas is required for IRMS. Reduction of water to hydrogen gas can be done with uranium or zinc [Preston 1992]. The different gases are separated by gas chromatography and analysed sequentially in continuous flow systems. For oxygen stable isotope ratio analysis, carbon monoxide is produced separately for testing as it is in traditional elemental analysis. With most of the procedure automatic, the sample preparation is greatly simplified. The cost of this analysis is less than €100 per element, and as little as €50 for only $^{13}C/^{12}C$ isotopic ratio analysis, depending on the service provider and the priority assigned to the sample.

The automated sample preparation widely implemented by an elemental analyser, as just described, is identical in most ways to the normal procedure of elemental analysis. However the difference is in the detection of the gases. It is not the amount of each element that is of interest, but the relative abundance of the stable isotopes of each element. The isotopic ratio mass spectrometer that is able to resolve the difference in mass between isotopes and detect their low relative quantities has become the basis of an established technique in specialist laboratories [Preston 1992]. Electron ionisation for the mass spectrometry is used. When considering carbon dioxide for example, the mass spectrometer must resolve the difference between $^{12}C^{16}O_2$ and $^{13}C^{16}O_2$. Full combustion must be achieved, otherwise $^{12}C^{16}O$ will interfere with the $^{14}N_2$ peak during nitrogen stable isotope analysis (known as isobaric interference) [Preston 1992].

Because only a small portion of the sample combustion gases are needed for IRMS, the spare carbon dioxide can be trapped and used for AMS ^{14}C isotopic analysis. Therefore only one method of sample preparation is required to serve both stable isotope analysis and ^{14}C radioisotope analysis. This is performed in a number of laboratories already and the need for further developments in the sample preparation phase of this technique is not a priority [Aerts-Bijma 2001, Olsen 2007, Fedi 2007]. The efficiency of combustion is discussed in **KBBPPS deliverable D4.1** (internal report, not publicly available). Sample preparation prior to introducing the sample into the analytical apparatus, such as acid washing, is another matter, not addressed in the aforementioned deliverable but discussed subsequently in this report.

As with ICP-MS, laser ablation can be used to prepare the sample for analysis [Ruso 2002]. A small amount of the solid sample is ejected with short bursts of a high energy laser, and the vapour of the sample combusted to give gaseous species on which IRMS can be performed [Moran 2011]. The technique is useful for historical artefacts, of which very little sample can be spared. For bio-based products this is not the case. With ACDV setting a precedent by requesting 100 g of sample for bio-based content verification, this means a great variety of analyses are possible with significant material to spare, though the cost implications of diverse and comprehensive analysis should be remembered. Where laser ablation will become useful is for large solid samples where sampling is an issue. If it is not possible to homogenise a material into a powdered form, then laser ablation can be used to sample the surface of the material, and a comparison made across a number of experiments. If the problem with sampling is purely about the reduction in size of a homogenous sample, then no further considerations would need to be accounted for. For layered composite materials that can be reduced in particle size, multiple experiments would serve to confirm the homogeneity of the prepared sample. Ultimately it would still be necessary to perform some sample preparation to obtain reproducible results.

The use of gas chromatography (GC) coupled with IRMS to give compound specific IRMS has been reviewed independently by several sources [Brenna 1997, Meier-Augenstein 1999, Schmidt 2004]. This is a powerful tool in the analysis of mixtures, possibly applicable to bio-based formulations. The first stage involves the gas chromatographic separation of the sample. Then a stable isotope analysis of each individual component can be conducted in sequence (**Figure 3.8**). By analysing each compound separately, stable isotope ratios remain distinct. Because the same source of an element (*e.g.* biomass) can result in an appreciable range of δ values, a mixture of compounds with elements from different sources will not lead to an easily calculable ratio of biomass to petroleum sources, as possible with ^{14}C analysis. Therefore separation of components would appear to be greatly beneficial.

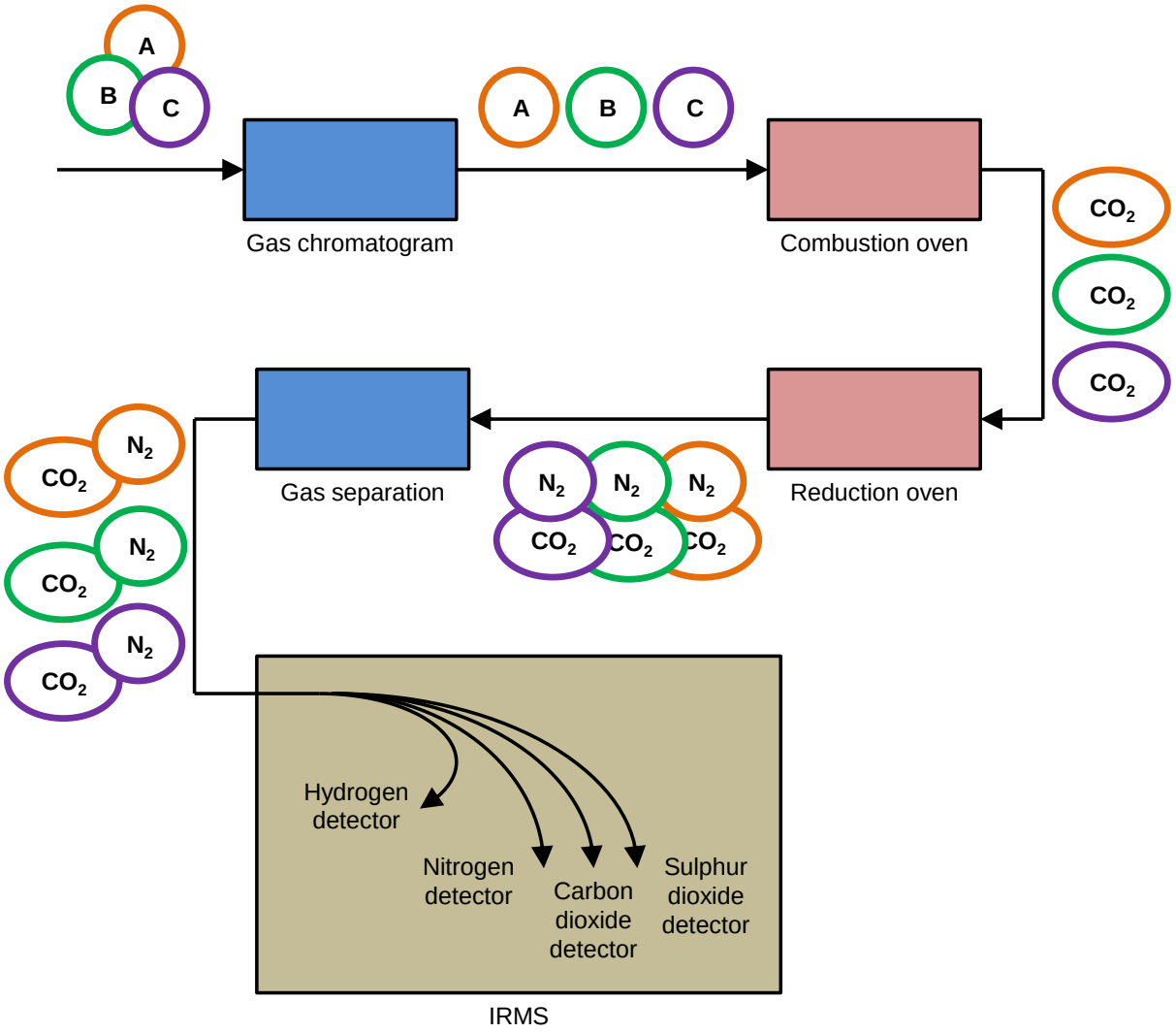

Figure 3.8 A schematic of compound specific, continuous flow isotopic ratio mass spectrometry, demonstrated for a three component sample.

There are disadvantages to compound specific IRMS. Firstly the components need to be volatile to be separated by the initial gas chromatography. When this is not the case, hyphenated EA-IRMS would need to be used without a fractionation pre-treatment. For the analysis of plastics, the largest bio-based product market, GC is not applicable. Simple solvent and lubricant formulations however could be analysed in this way, given all the components are compatible with the analysis. Thermally sensitive compounds are not compatible, for it would be their decomposition products that would ultimately be analysed, and not the original component of the sample. An additional source of error in compound specific IRMS is the overlapping of signals, not just of the gaseous combustion products, but of the sample components as they are fractionated on the first GC column. [Blessing 2008, Yanik 2003]. Method development or simply a longer column may be required depending on the complexity of the sample.

3.3.4 Other analysis techniques

The stable isotope ratios of elements in water and carbon dioxide can be inferred using infra-red (IR) spectroscopy [Griffith 2006, West 2010, Zhao 2011]. Using cavity ring down spectroscopy, a form of laser absorption spectroscopy, the process seems to be suspectible to errors and not as reliable as other isotope ratio detection techniques [West 2010].

A more viable technique for the isotopic analysis of hydrogen in certain molecules is nuclear magnetic resonance (NMR) [Martin 1982]. The use of ^{2}H NMR spectroscopy with decoupled proton signals is able to deduce the $^{2}H/^{1}H$ isotopic ratio present in ethanol samples. As a result the origin of the ethanol can be inferred (**Figure 3.9**). A small difference seems to be present between C_3 (yellow bars in **Figure 3.9**) and C_4 (green bars in **Figure 3.9**) plant derived ethanol, but larger studies have revealed that although C_4 plants have a lesser disinclination towards deuterium on average, the ranges of $\delta^{2}H$ overlap [Vitzthum von Eckstaedt 2012]. What is more useful is the seemingly significant difference between petroleum derived ethanol (red data bars in **Figure 3.9**) and bio-ethanol. In terms of sample preparation, none is required for the aqueous ethanol samples for which this analysis is routinely used. The limitation of this technique is the required purity of the sample. Complex mixtures containing components in low concentration will not have distinct NMR signals from which to calculate isotopic ratios.

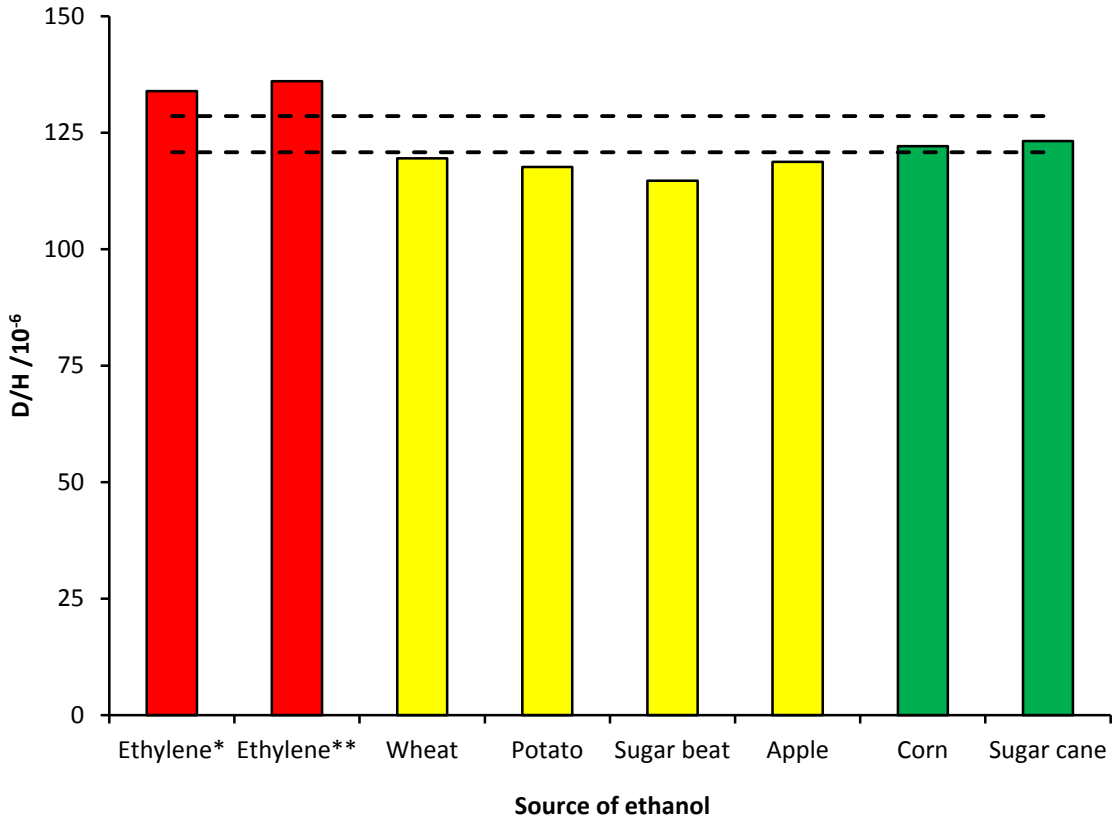

Figure 3.9 Ratios of deuterium to hydrogen in ethanol from different sources. *Production described as 'ethylene contact'. **Ethanol by sulphuric acid hydration of ethylene.

3.4 Sample preparation standards for elemental and isotopic analysis

3.4.1 European standards

There are a variety of product specific standard test methods covering sampling, sample preparation, and the determination of elemental composition and bio-based content. Of those, CEN has produced the following documentation for European needs. Relevance to sample preparation is highlighted, although the original intention of the standard is often for some type of analysis or processing unrelated to bio-based content.

An attempt has been made to tie in these standardised sample preparation protocols with the probable choice of total bio-based content determination method that will be implemented in the future. It is likely that stable isotope analysis (and elemental analysis) will be used for this purpose, but few standards are specifically designed to address this technique. Sample preparation standards are often non-specific and designed to be broadly applicable.

EN 14780 Solid biofuels – sample preparation

European standard **EN 14780** is designated as suitable for the preparation of solid biofuels (wheat straw, wood chips, *etc*.) for various tests, including chemical composition. The definition of sample preparation in this document is taken as the reducing of sample size to more manageable, suitable amounts. **EN 15442** covers the original sampling of the solid biofuels (not covered here).

The issue of moisture is addressed, and will be of considerable importance for most varieties of bio-based product. Oven drying at 313 K is recommended, followed by 24 hours of air drying in storage. Water content lost in the drying process should be recorded for mass balance calculations. Such mild drying conditions would fail to remove water from surfactant formulations for example, although it is only intended within this test method as a means of minimising errors when reducing sample size. The following description of moisture content determination in the European standard **EN 14774-3** (as reviewed later) seems more thorough for pre-analysis sample preparation.

EN 15443 Solid recovered fuels – Methods for the preparation of the laboratory samples

Sample preparation of solid recovered fuels is defined by **EN 15443**. The process of sample preparation is suggested in this document to be the reduction of size of the sample into portions of suitable particle size without changing the chemical composition of the sample. Note that these processes are only applicable to solids, for homogenous fluids need no such preparation, and heterogeneous solid-liquid or liquid-liquid articles are not addressed. An upper limit of particle size is defined according the size of the original sample. Milling to reduce particle size must be conducted in such a way that volatiles are not lost.

Apparatus suitable for sample division into useful portions for analysis are set out in **EN 15443**, including rotary dividers for smaller particles and shovels for large biomass samples. This is not relevant for bio-based products supplied as commercial consumer articles due to their manageable size (a bottle of cleaning fluid, a canister of motor oil, or some plastic packaging material for example). Business supplies of bio-based materials or chemical intermediates will be distributed in much larger volumes, and batches may need to be sampled in this way. Particle size reduction methods with cutting mills are defined, as are the sieves used to ensure the correct particle size has been achieved.

Drying of the sample prior to sample division and particle size reduction is also addressed, and is conducted in an oven at 313 K. Constant weight needs to be achieved after air drying at room temperature for 24 hours, which can be performed after oven drying. The recommended drying procedure is a consequence of the nature of the materials for which this standard was developed. The preparation of surfactant samples from their aqueous solutions will require much more efficient drying methods.

EN 15413 Solid recovered fuels. Methods for the preparation of the test sample from the laboratory sample

The **EN 15413** standard test method follows on from **EN 15443** by further processing fuel samples in preparation for chemical analysis. Again the purpose is for sample size and particle reduction. The order of pre-treatment follows this order:

1. Fraction separation
2. Drying
3. Particle size reduction
4. Homogenisation
5. Sub-sampling

The emphasis of **EN 15413** is the decision making processes that concerns sample preparation on a case-by-case basis. The plan will vary slightly for each sample. For drying of samples, it is recommended that water should be removed that would otherwise hamper other pre-treatment steps, such as particle size reduction. Therefore complete water removal is not compulsory or even encouraged. For accurate water content determination other standard test methods must be used such as **EN 14774-3** and **ISO 15512**. Air drying of the sample is not recommended when "*time is critical*", which would seem to undermine the pre-treatment methodology when higher temperature drying methods cannot be applied. For bio-based product compositional analysis, the moisture content may have a key role, especially when many such products are aqueous solutions. The difference between organic and inorganic content, and how to treat the latter in total biomass content determination, will be decided to a large extent by the approach adopted in addressing the water present in formulations. Oven drying at 378 K is addressed, and recommended when auto-ignition can be

avoided. Freeze drying was not included as an option for drying samples, as this is only effective for samples with a high water content.

Particle size reduction methods vary between product types, with freeze crushing recommended form plastic containing materials (with obvious relevance to bioplastics). In order to conduct freeze crushing the sample contained in a polyethylene container and immersed in liquid nitrogen for 10 minutes. The sample is then broken with a hammer, unless a more appropriate tool is recommended by the supplier. Certain plastics, especially films, may not benefit from freeze crushing and so cutting with scissors, or a cutting mill, is used instead. The sample can be frozen with liquid nitrogen prior to cutting.

In between processing, the sample must be stored correctly. Prior to elemental analysis the sample is to be stored at 277 K in a (non-PVC) plastic container. Contamination from the air (especially dust) must be avoided, as should the loss of material by evaporation. The sample size must be at least 0.1 kg just for CHN elemental analysis, increasing to 0.8 kg for more complete analyses. Being that this case study is specific to recovered fuels, these seemingly excessive amounts may not translate to bio-based products.

EN 14774-3 Solid biofuels - Determination of moisture content - Oven dry method - Part 3: Moisture in general analysis sample

A major part of sample preparation for bio-based content analysis will be the removal of water, or at least a moisture content determination so that water can be factored into subsequent calculations. For solid biofuels the latter is covered by **EN 14774-3**. This procedure for moisture content determination is as follows:

1. A suitable vessel (including its lid) is dried at 378 K and cooled in a desiccator.
2. A minimum of 1 g of the sample is placed uniformly in the vessel.
3. The open vessel and contents (and lid separately) are heated at 378 K until constant in mass. This is defined as the point at which an additional hour of drying at 378 K does not result in more than 0.001 g of mass loss. This is expected to take no more than 3 hours.
4. The lid is replaced while still in the oven, and the sealed vessel placed in a desiccator to cool to the ambient temperature.
5. A record of the mass loss is made as soon as possible, especially for hygroscopic materials (this will be important for surfactants in broader bio-based content analysis procedures).

The procedure is robust and checks are in place to ensure reproducibility and repeatability. However this moisture determination only measures the removable (labile) water under the conditions of the drying oven and not the absolute water content. A benefit to mild drying conditions such as this method is that latent water content is not inadvertently recorded which would be worse than not removing all the free water in the sample. This procedure

is suitable for eliminating variables that may affect further sample preparation, but not ensuring complete dryness. That may have some implications on mass balance calculations for the purpose of total bio-based content determination. Surfactants for example are very hygroscopic, remaining as a gel even after freeze drying. Additionally, this methodologies risk errors as a result of volatile components, such as fragrances, being lost during the drying process.

EN 15104 Solid biofuels - Determination of total content of carbon, hydrogen and nitrogen – Instrumental methods

Remaining with solid biofuels, the determination of their elemental composition, limited to carbon, hydrogen, and nitrogen, has been standardised as **EN 15104**. The procedure should now be familiar: The sample is combusted to yield carbon dioxide, water, and nitrogen. Intermediate nitrogen oxides are reduce to elemental dinitrogen and any hydrogen sulphide or hydrogen halide gases are fully converted to water for accurate hydrogen analysis. Certified calibration substances are required for accurate results.

Sample preparation is defined through reference to other standards. Particle size should be a maximum of 1 mm (remembering the relevance to solid biofuels) as defined in **EN 14780**. Moisture content must be determined with **EN 14774-3** as this will create significant errors in elemental analysis if water is introduced as an unknown variable. With regards to the actual test sub-sample, all that is suggested is that the sample material is placed in a suitable capsule for the analysis. This is likely to be a crimped tin vessel.

The actual apparatus of elemental analysis is not defined, instead a list of minimum requirements is provided. These centre on assurances over complete combustion and reduction where necessary to provide quantitative amounts of carbon dioxide, water, and nitrogen. Results should be reported on the basis of dry weight, and the analysis repeated so that a reliable mean average can be produced.

EN 15289 Solid biofuels — Determination of total content of sulphur and chlorine

This standard methodology reports on the analysis of chlorine and sulphur in biofuels. Manual preparation (bomb calorimeter combustion is preferred), automatic preparation (elemental analyser), and spectroscopic/spectrometric methods (X-ray fluorescence and ICP included) are all covered by **EN 15289**.

All that is required in sample preparation is that any particulate matter does not exceed 0.001 m in diameter. Bomb combustion requires that the sample is pressed into a pellet, as is standard practice for bomb calorimetry. The combustion is performed, as is typical, under 30 standard atmospheres of oxygen. The gaseous products of chlorine and sulphur combustion (hydrogen chloride and sulphur oxides) can be trapped as aqueous solutions for subsequent analysis.

EN 15440 Solid recovered fuels – Method for the determination of biomass content

The determination of the bio-based content of solid recovered fuels has been established as **EN 15440**. Three methods of analysis are described: 'selective dissolution' of biomass by sulphuric acid and hydrogen peroxide, manual separation by visual inspection, and ^{14}C isotopic abundance. This may result in the amount of biomass being classified as a function of carbon content, as a mass balance, or by energy content depending on the analysis conducted. Of course this assessment is based on the proportion of biomass present, and not any downstream products made from renewable feedstocks of the type expected to be present in bio-based products. As such the scope and purpose of this standard is not fully appropriate, but some aspects of the sample preparation can be translated to alternative methods of bio-based content determination. Alignment as much as possible with existing protocols would be preferable of course until the point where this is detrimental to new methods of total bio-based content.

The intention with selective dissolution is that any biomass will be broken down with these treatments, and then the remaining material will represent purely petroleum derived materials. Selective dissolution does not work for a variety of materials (*e.g.* non-biodegradable bio-based plastics and biodegradable fossil based plastics) and is of limited usefulness for bio-derived materials (rather than actual biomass as originally intended). Sample preparation is deferred to **EN 15442** and **EN 15443**. Drying at 378 K is required before dissolution of the biomass with sulphuric acid and then hydrogen peroxide.

Manual separation is only suitable for obviously heterogeneous samples consisting of particles of size greater than 0.01 m. Again the sample must be dried in an oven and cooled in a desiccator. The final methodology is a ^{14}C isotopic abundance analysis. Any carbonate content is removed with sodium hydroxide solution. Combustion of the prepared sample is possible in a calorimeter, a tube furnace, or other combustion apparatus. The latter is more amenable for the additional analysis of stable isotopes, in which, post-combustion, the majority of the sample's carbon dioxide can be redirected for the AMS intended of this test methodology. Complete combustion is required, and the carbon dioxide can be trapped cryogenically. This is converted to graphite with an iron catalyst to give an analysable material. Full procedures for radiocarbon analysis are available in **ASTM D6866**. The chief limitations of ^{14}C isotopic abundance analysis are described as (1) precision uncertainty created after nuclear bomb tests, and (2) the small number of AMS equipped labs. Error in AMS bio-based carbon content analysis is said to be ±3%, according to **ASTM D6866**.

CEN/TS 16137 Plastics - Determination of bio-based carbon content

The previous standards have focused on biofuels and not bio-based products. Recent developments have described bio-based plastic test standards, with technical specifications such as **CEN/TS 16137** created for this purpose. This document relies on ^{14}C content measurements by proportional scintillation-counter method (PSM), beta-ionisation (BI) and accel-

erator mass spectrometry (AMS). Concerns over the accuracy of the first two methods means this discussion will equate ^{14}C content analysis with radioisotope detection by AMS. In fact PSM was dropped from **ASTM D6866** for this reason, and is not present in the current verion (D6866-12). Essentially the sample preparation is the same as other methods (**EN 15440** and **ASTM D6866**). Inorganic carbon is removed with sodium hydroxide solution before combustion to carbon dioxide. Once again combustion of the prepared sample is possible in a calorimeter, a tube furnace, or a combustion apparatus. Other pre-treatments such as drying are not discussed. Sampling is deferred to other unspecified standards.

Overall this technical specification just reiterates the bio-based carbon content method described by **ASTM D6866** and **EN 15440**, but limits itself to plastics. The American standard **ASTM D6866** is vague in terms of sample preparation prior to the necessary carbonate removal and combustion steps also, for this of course is the responsibility of other standards that describe general sample preparation methods for a variety of subsequent analytical methods. Instead **ASTM 7026** should be referred to for this purpose (described subsequently).

ENV 12142 Method for determination of stable hydrogen isotope ratio (2H/1H) of water from fruit juices, using isotope ratio mass spectrometry

Standards specifically designated for stable isotope analysis are limited. The prestandard **ENV 12142** considers the hydrogen stable isotope ratios (δ^2H) found in the water of fruit juices by IRMS. This can reveal the difference between products made from concentrate and unprocessed fruit juices. As always the difference in mass between H_2 gas consisting of two protium atoms ($^1H_2$), and a protium atom and a deuterium atom ($^2H^1H$) is the basis of this analysis. Dideuterium ($^2H_2$) is sufficiently obscure to not skew the analysis.

The production of hydrogen gas from water is performed with a technique that requires separate sample preparation (water reduction to hydrogen gas) and a subsequent isotopic analysis (IRMS) stage, not the common EA-IRMS hyphenated technique already explained. The full sample preparation begins with the centrifugation of the sample to remove solid fruit matter, then distillation is needed for quantitative water reclamation. This separation, of fruit solids and water, is not directly applicable to the analysis of bio-based products, but in cases where homogeneity of the sample cannot be assured, sampling will be biased. In this instance separate isotopic analyses of distinct formulation components may become necessary. This issue is addressed further in **ASTM D7026**.

The reduction of water to hydrogen gas can be performed with either uranium or zinc containing furnaces, described in greater detail within **ENV 12142** itself. The gas is collected in a suitable container and transferred to the IRMS apparatus. The use of EA-IRMS apparatus in which the sample could be converted into hydrogen and the hydrogen isotopic ratio of the hydrogen gas analysed, as a concerted process, would appear to be a simplification of the procedure in **ENV 12142**. The following discussion (*section 3.5*) describes the work of

several test centres that operate EA-IRMS for stable isotope analysis. It is prevalent enough to be a viable option, and seemingly reliable.

ENV 12140 Method for determination of stable carbon isotope ratio ($^{13}C/^{12}C$) of sugars from fruit juices, using isotope ratio mass spectrometry

Similar in context to **ENV 12142**, **ENV 12140** provides a standard test method for carbon stable isotope analysis of the sugars in fruit juices. The sample is centrifuged, and addition of calcium hydroxide to the solid is used to selectively dissolve the sugar content of the sample. The sample is freeze dried to give a powder for analysis. The conditions under which the sample should be dried were not given. Unlike **ENV 12142**, the analysis of the prepared sample in **ENV 12140** is by an online (*i.e.* in sequence) EA-IRMS set-up. Combustion to carbon dioxide allows for the determination of $\delta^{13}C$. The reproducibility of the procedure is said to be high (data supplied within the standard).

ENV 13070 Fruit and vegetable juices - Determination of the stable carbon isotope ratio ($^{13}C/^{12}C$) in the pulp of fruit juices - Method using isotope ratio mass spectrometry

The principle behind **ENV 12140** is used in this related pre-standard **ENV 13070**. It too deals with stable carbon isotope ratios, but this time of the pulp of fruit juices. A centrifuge is used to separate the solid fruit material (pulp) from the water. Lipids are removed with acetone, otherwise a large variability in $\delta^{13}C$ values results. Combustion of the pulp is subject to the usual constraints (full combustion required and all NO_x needs to be reduced) using the method in **ENV 12140** with automated EA-IRMS.

3.4.2 Other standards

The closest procedure to a standardised total bio-content analysis method currently in use is the method set out in **ASTM D6866**, although this is limited to carbon analysis. All existing bio-based product certification schemes are reliant on it. This gives a direct measurement of the bio-based carbon content of the sample and is covered in greater detail within other reports emerging from the KBBPPS research project (**KBBPPS deliverable D3.6**, due to be published in 2015). The later stages of sample preparation required for isotope analysis have been addressed in discussions presented thus far, as well as the hyphenation of accelerated mass spectrometry (AMS) for $^{14}C/^{12}C$ ratio determination with stable isotope analysis technique. Prior to combustion **ASTM D6866** gives no guidance on sample preparation. Nevertheless demonstrations of the protocol seem robust [Norton 2006, Norton 2007].

To appreciate the types of sample preparation implemented in laboratory analysis, actual accounts of these procedures need to be recounted, and such discussion follows in *section 3.5*. In this section the focus of discussion is on sample preparation, prior to broader stable isotope analysis, and consists of other American (ASTM) and international (ISO)

standards. The relevance of **ASTM D6866** will be revisited when appropriate after an initial summary, especially when concerning discussions on the related standard, **ASTM D7026**.

ASTM D6866 Standard test methods for determining the biobased content of solid, liquid, and gaseous samples using radiocarbon analysis

Simply put, the outcome of **ASTM D6866** is a percentage bio-based carbon content, as a portion of the total organic carbon content of the sample. This standard test method is being used for the analysis of bio-based products, and has been in operation since the implementation of the USA Farm Security and Rural Investment Act of 2002 [BioPreferred 2014]. In recent versions of the document, two methods (reduced from the original three) are described for measuring the proportion of ^{14}C in a sample of graphite (for AMS) or benzene (for liquid scintillation counting, LSC). Both require the conversion of the sample to carbon dioxide. The sample preparation before combustion is covered in **ASTM D7026**.

Combustion of the sample is performed by the following technique provided in **ASTM D6866**. This procedure is the recommended methodology because of its affordability and extensive use worldwide. As little as 1 mg of carbon is needed, but must be quantitatively recovered as carbon dioxide so that the subsequent radiocarbon analysis is representative.

The sample material is transferred into an appropriately sized quartz tube. For materials containing volatile components, the sample within the tube shall be frozen with liquid nitrogen prior to evacuation of the ambient atmosphere. The sample is combusted in a temperature controlled furnace at 1173 K for up to 4 hours under an oxygen atmosphere. After combustion, the quartz sample tube needs to be carefully broken within a flexible hose portion of a “tube cracker” assembly adapted to a manifold. The sample carbon dioxide is cryogenically frozen with liquid nitrogen and transferred to another vessel. The sample is distilled to remove residual water using a dry ice/alcohol slurry maintained at about 197 K. The carbon dioxide gas is released and immediately condensed. The recovery efficiency is determined using the ideal gas law relationship. The sample shall be transferred to a borosilicate break seal tube for storage and delivered to an AMS facility for analysis of the $^{14}C/^{12}C$ isotopic ratio.

ASTM D7026 Standard guide for sampling and reporting of results for determination of biobased content of materials via carbon isotope analysis

American standard **ASTM D7026** has been written to develop the sample preparation required for $^{14}C/^{12}C$ radioisotopic analysis beyond that presented in **ASTM D6866**, and the principle can be extrapolated to related $^{13}C/^{12}C$ stable isotope analysis methods. The standard describes the use of cryogenic grinding for solids if the sample needs to be reduced in size. Up to 0.01 g of sample, once combusted to CO_2, is needed for AMS. Liquid nitrogen must be used to cool the sample for grinding, for dry ice may saturate porous materials with

carbon dioxide adding major errors to the analysis. Ultimately it is desirable for particles below 0.00025 m in diameter to be obtained.

Fluids must be homogeneous. Emulsions are classified as homogeneous if stable indefinitely. If a liquid sample is a heterogeneous mixture (identified from its turbidity) then it should be centrifuged. The resulting liquid and solid fractions should be analysed separately. With a mass balance the results of the separate analyses can be proportioned correctly to confidently obtain a reliable value of bio-based carbon content for the whole formulated product.

A research paper has evaluated the latter stages of sample preparation in the context of **ASTM D6866** to reduce the introduction of errors that latter affect radioisotope analysis, but also applicable to stable isotope ratios [Culp 2009]. Combustion in bomb calorimetry apparatus was investigated, and although it is effective in combusting the material, it is not amenable with continuous analysis. As with other research articles, the acid washing stages required for carbonate removal need to be implemented with care, but ultimately are manageable.

ASTM E1757 Standard practice for preparation of biomass for compositional analysis

A standard has been developed that emphasises the importance of drying the sample as part of the sample preparation step, with this standard focussing on biomass analysis. In **ASTM E1757** three drying methods are described: air drying, low temperature oven drying, and freeze drying. In addition to **ASTM E1757** a document from the National Renewable Energy Laboratory (NREL) of the USA reiterates these preparatory measures for compositional analysis; essentially their interpretation of **ASTM E1757** [Hames 2008]. As with **ASTM E1757**, the scope of this NREL document is primarily to cover biomass preparation. However the methodology is also applicable to solid materials derived from biomass (*i.e.* bio-based products). The aim is to provide samples small in size and also dry. Therefore dry (<10% water content) particulate matter (<0.00085 m diameter) requires no pre-treatment of this nature.

Air-drying is used for large quantities of biomass in order to reduce the moisture content below 10 wt%. This is applicable to large quantities of wood samples for example, and accordingly is not so appropriate for bio-based products where water content is likely to be low (*e.g.* PLA plastic or lubricants). Whereas biofuel analysis standards explain oven drying as being conducted at 378 K, a temperature of 318 K is advocated here, for up to 2 days when air drying is not sufficient. Again the emphasis is on biomass, hence these mild conditions and large sample size description (up to 20 g). Oven drying ends when less than 1% mass loss is observed across a duration of one hour. Finally freeze drying for very wet biomass that would otherwise be at risk of microbial degradation is recommended. This seems to be the most viable procedure of bio-based products in general. The freeze drying apparatus used needs to be able to achieve temperatures of 223 K and a pressure of <1 torr. An-

other reference gives 255 K as the maximum temperature necessary when operating at a lower pressure (10^{-1} mbar, equivalent to 0.075 torr) [Brock 2010].

The drying is followed by milling to make the sample particulate small in size. The associated quality control is highly mathematical and will not be recounted in depth here, only to reiterate that a knife mill is used to reduce the particle size until appropriate for subsequent tests.

ASTM D7459 Collection of integrated samples for the speciation of biomass (biogenic) and fossil-derived carbon dioxide emitted from stationary emission sources

It is true that this standard, **ASTM D7459**, is perhaps not entirely representative of a typical bio-based product analysis, but it does begin to address the sample preparation of gases, often out of the scope of other standards in this area. For the case of carbon dioxide this gas is already in the form needed for carbon isotope analysis. The gas is collected and passed through a water condenser to remove water moisture. The actual carbon dioxide is stored as a gas. A gas bag suitable for this purpose must not be permeable to other gases. Aluminium lined gas bags can be used for carbon dioxide storage. A cryogenic trap to solidify the carbon dioxide would be preferable for transportation, and in the case of bio-based products to help retain a representative sample.

This standard, **ASTM D7459**, is complimentary to **ISO 13833**, which is familiarly entitled *Stationary source emission – Determination of the ratio of biomass (biogenic) and fossil-derived carbon dioxide – Radiocarbon sampling and determination*. In this document, a procedure for trapping the carbon dioxide collected from stationary emission points is described that culminates in the formation of a carbonate solution as an alternative to collection in a gas bag. For the former an excess of an alkaline carbonate solution is needed.

Methods for trapping gaseous materials is applicable to bio-based product analysis, indeed **ASTM D7459** and **ISO 13833** have been implemented for the purpose of biogenic composition determination. To convert a carbonate solution to carbon dioxide for carbon ratioisotope analysis, acid is introduced after degassing to remove atmospheric carbon dioxide. The resulting gas is passed through a dry ice-acetone cold trap to remove water vapor. Then the purified carbon dioxide is frozen in liquid nitrogen. The methodology is applicable to organic gases too, given that they are combusted to carbon dioxide first in bomb calorimetry apparatus.

ASTM D5291 Standard test methods for instrumental determination of carbon, hydrogen, and nitrogen in petroleum products and lubricants

The standard **ASTM D5291** was the first to cover the combined, simultaneous determination of carbon, hydrogen, and nitrogen. Upon combustion the familiar gaseous products of carbon dioxide, water, nitrogen oxides, hydrogen halides, and sulphur oxides are formed.

Four different standard test methods are given for the separation of these gases for individual analysis (*e.g*. for stable isotope ratio analysis). The emphasis of the standard on lubricants brings discussion more into line with the purpose of this report; that being the sample preparation of bio-based products for complete biogenic analysis.

In the first test method the products of combustion are first treated with calcium oxide to remove sulphur containing gases. Between 0.05 g and 0.20 g is required for solid samples. Liquid samples are absorbed into magnesium oxide within their crimp sealed copper vessel. The use of magnesium oxide reduces the issues experienced with the combustion of solvents, such as evaporation causing incomplete combustion and explosion. Copper is used in the reductive oven to remove oxygen, reducing nitrogen oxides to dinitrogen gas. Then sodium hydroxide can be used to remove carbon dioxide and finally magnesium perchlorate will eliminate water. The remaining nitrogen gas can be analysed pure. This test method is not of the automated type routinely in operation, and so aside from the use of an absorbent for liquid samples, and other aspects that are repeated as parts of subsequent analyses, the rest can be disregarded as the possible basis of a horizontal, high throughput method of stable isotope analysis.

For the next test method, the more familiar elemental analysis set-up, only 0.002 g of sample is required, with a combustion oven set at 1248 K and the copper packed reduction oven at 913 K. The purified combustion product gas stream (minus sulphur oxides and extra oxygen as before), consisting of carbon dioxide, water and nitrogen is flushed into a mixing chamber. After homogenisation (*i.e*. mixing), the sample passes through a GC column to create the required separation. Use of a calibration standard is requested rather than using absolute values determined directly from the sample, as is current practice.

The third gas generation and separation method requires 0.005 g of sample placed and sealed within a tin capsule, but is in essence very similar to the methodology just described. The combustion oven operates at 1293 K and the reduction occurs at a temperature of 923 K. The separated gases are measured by using thermal conductivity, and a standard required every ten samples to maintain accuracy.

The final test method requires 0.003 mg of sample in a tin vessel, crimped to seal. An absorbent is suggested for volatile liquid samples, and combusted in a reactor at 1323 K. To achieve quantitative combustion the primary gaseous products are reacted over chromium trioxide and cupric oxide in the oxidising combustion oven. Copper is used to produce nitrogen from the nitrous oxides. The column separation occurs anywhere between 323 K to 393 K, depending on the apparatus. The gases elute in the following order: nitrogen, then carbon dioxide, and finally water for the detection of nitrogen, carbon, and hydrogen elements respectively.

ASTM D7455 Standard practice and sample preparation of petroleum and lubricant products for elemental analysis

Elemental analysis is covered specifically for lubricants by **ASTM D7455**. Bio-based lubricants are developing strongly within the bio-based products sector and this standard test methodology should be directly translatable to this sub-set of articles. Some general good laboratory practice is described; including minimising any loses of volatile compounds with suitable containment, and contamination control.

The sample preparations described may be for elemental analysis by ICP-AES, atomic absorption spectrometry, or X-ray fluorescence, although these techniques do not necessarily require any manual sample preparation aside from dissolution of the sample in a solvent. The elemental analysis of lubricants requires combustion in order to calculate the amounts of carbon, hydrogen, and nitrogen present, as familiar from previous discussions, and does not require any special sample preparation either.

ISO 10210 Plastics – Methods for the preparation of samples for biodegradation testing of plastic materials

The purpose of this standard, **ISO 10210**, is the unification of sample preparations prior to biodegradation studies on plastics. It is widely applicable across different varieties of plastic materials. As the major bio-based product market sector this is of significant importance. A strong focus of **ISO 10210** is achieving the homogeneity of the sample, both in sample size and surface area. This is not vital to the determination of isotopic composition as the sample is combusted prior to the analysis. What is important however is achieving a representative sample (through sampling) and then to apply this representative portion of the bio-based product in analysis. When a non-particulate solid product is to be analysed, a representative portion should be cut free of cubic dimensions between 0.005 m and 0.010 m. Many plastic products, such as carrier bags, cups and cutlery, can simply be cut to size with a knife or scissors.

Milling to reduce particle size and improve homogeneity is recommended, with either solid carbon dioxide or liquid nitrogen used as a refrigerant. In light of the scope of this work, only the use of liquid nitrogen would be compatible in this sense, with the unnecessary risk of introducing foreign carbon dioxide to the sample prior to isotope analysis best avoided (liquid nitrogen can condense CO_2 from the air). Otherwise there is a risk of altering the stable isotopic ratios of carbon in the sample. It might be argued that liquid nitrogen would have the same effect on nitrogen isotope analysis, but nitrogen is chemically inert, whereas carbon dioxide is not and will chemically react as well as dissolve and absorb onto materials. If nitrogen isotope analysis is being conducted separately then solid carbon dioxide would be an acceptable refrigerant.

The success of the sample preparation is gauged visually, with discolouration and obvious impurities indicators that the sample has been chemically altered. This is obviously not a comprehensive approach, but is unlikely to produce a false negative. To maximise the probability of a successful, representative fractionation, as few stages as possible should be implemented. Milling and drying would seem to be sufficient in the majority of cases.

ISO 1795 Rubber, raw natural and raw synthetic – Sampling and further preparative procedures

Milling is used as the primary method of sample preparation for rubber materials as described by **ISO 1795**. A roll mill is required for rubbers, generally operating at 323 K. Interestingly the sample is subjected to a mass balance before and after sample preparation to ensure the loss of sample volatiles is not significant. If full reclamation of the material is achievable after other types of milling too, then this would appear to be a very beneficial undertaking as a quick form of quality control. No method for the confirmation of homogeneity after milling was described.

ASTM D297 Standard test methods for rubber-products chemical analysis

This test methodology (**ASTM D297**) is for the determination of rubber polymer content within a sample. A detailed sample preparation method is described, beginning with milling of the sample to reduce sample size. If suitable milling machinery is not available, it may be able to process the sample by other means, such as cutting with scissors until the material can be passed through a 0.0014 m sieve. This sample preparation would be applicable to all bioplastic films. The entire sample must be reduced in size and passed through the sieve to avoid inadvertent fractionation and therefore biased, disproportional sampling.

The chemical analysis tests that follow the suggested sample preparation vary in relevance. Amongst them number of specific chemical identification tests are described such as the quantification of magnesium oxide content through its reaction with diammonium hydrogen phosphate to give magnesium pyrophosphate. With the availability of ICP analysis, this compound specific analysis on metal salts is not necessary for the determination of total bio-based content *via* elemental analysis.

TAPPI T257 Preparation of wood for chemical analysis

Wood analysis is performed on milled samples before solvent washing. This removes chemical components not considered to be wood. Acetone then water is used to extract resin, tannins, and sugars amongst other things. In order to do this a Soxhlet extractor is used. On review it seems to be unlikely that this fractionation will be generally applicable to bio-based product analysis. In some specific cases it might be helpful to remove solubles from a product, perhaps the dyes or additives present in a bioplastic. If so then the standardised Soxhlet extraction method demonstrated in **TAPPI T257** may be of broader utility.

ISO 5089 Textiles - Preparation of laboratory test samples and test specimens for chemical testing

Sample preparation for textiles has been described by **ISO 5089**. As always it is important to withdraw a portion of the sample that is representative of the proportions of components in the entire material. Relevance to bio-based products may include fabrics woven from synthetic polymers, possibly of a renewable origin. Fabrics can simply be cut to size following the guidelines in **ISO 5089**.

Following on from **ISO 5089**, **ASTM D620**: (entitled *Standard test method for quantitative analysis of textiles)*, offers more detail for the sample preparation of textiles, and in the context of analytical procedures. Mechanical separation followed by drying is described. Drying is conducted at 378 K for a duration of 90 minutes, and a mass balance reported to the nearest milligram. No protocol to ensure complete dryness is given.

ASTM D1762 Standard test methods for chemical analysis of wood charcoal

Sample preparation of charcoal according to **ASTM D1762** consists of air drying and moisture content determination. The sample is ground into a powder, and drying is conducted at 387 K in an oven. Particle size is defined in the document, which may be achieved with milling apparatus. The necessity for the entire sample to be processed without losses is reiterated, this being a feature of previous standard test methods.

3.5 Bio-based product analysis case studies: Stable isotopic ratio determination

3.5.1 Multi-isotopic ratio analyses

From the introductory description of stable isotopes and their analysis, it seems that the combination of different elements in a multi-isotopic testing regime would be the most effective use of this technology for the purpose of bio-based content analysis. Not enough information can be gained from the stable isotopic ratio of one element, or indeed the radio-isotope analysis of carbon alone. Sample preparation for this purpose has been suggested through the review of existing standards. The following excursion through the relevant scientific literature will serve to justify the focus on stable isotope ratios, proving the value of multi-isotopic ratio analysis, and assist remaining discussions on what pre-treatments will be necessary and what effect they may have when put into practice.

In one example of a multi-isotopic analysis, the combination of carbon, hydrogen, and oxygen stable isotope data from IRMS has been used to identify the origin of ethanol as an alternative to deuterium abundance calculations from NMR spectroscopy [Ishida-Fujii 2005, Yamada 2007]. Differences in the $^{13}C/^{12}C$ isotopic ratios found in C_4 carbon fixing metabolising plants to that of C_3 plants and fossil based chemicals means that stable carbon isotope analysis is only adequate for chemicals made from certain plants but not others (**Figure 3.10**). When $^{13}C/^{12}C$ isotopic ratios are consistent with petroleum derived materials, this in fact is not indicative of either a petroleum derived chemical or a bio-based product. So the use of other stable isotopes is beneficial to cover this limitation. It was found that the D/H isotopic ratio is still useful, but plotting a two dimensional graph of δ^2H versus $\delta^{18}O$ clarifies the assignment of bio-ethanol versus synthetic ethanol produced from petroleum sourced ethylene [Ishida-Fujii 2005]. Oxygen isotopic analysis proved quite revealing, separating fossil-based and fermentation ethanols (**Figure 3.11**). Synthetic ethanol is made by the hydration of ethylene, and so the oxygen content originates in molecules of water. The oxygen atoms that are incorporated into bio-ethanol molecules originate in fresh plant carbohydrate but those atoms are known to fully exchange with water [Ehleringer 1989, Epstein 1977]. Therefore ultimately the original oxygen source is the same for both ethanol products (fossil and plant derived). The greater abundance of the heavier oxygen isotope (^{18}O) in plants is due to this exchange between the labile hydroxyl groups of sugars (and its polymers) with water, and then vapour pressure fractionation of water in the plant [Ehleringer 1989]. This means the more volatile, lower mass water isotopomers leave the plant at a faster rate, enriching the value of $\delta^{18}O$ in downstream bio-based products such as ethanol.

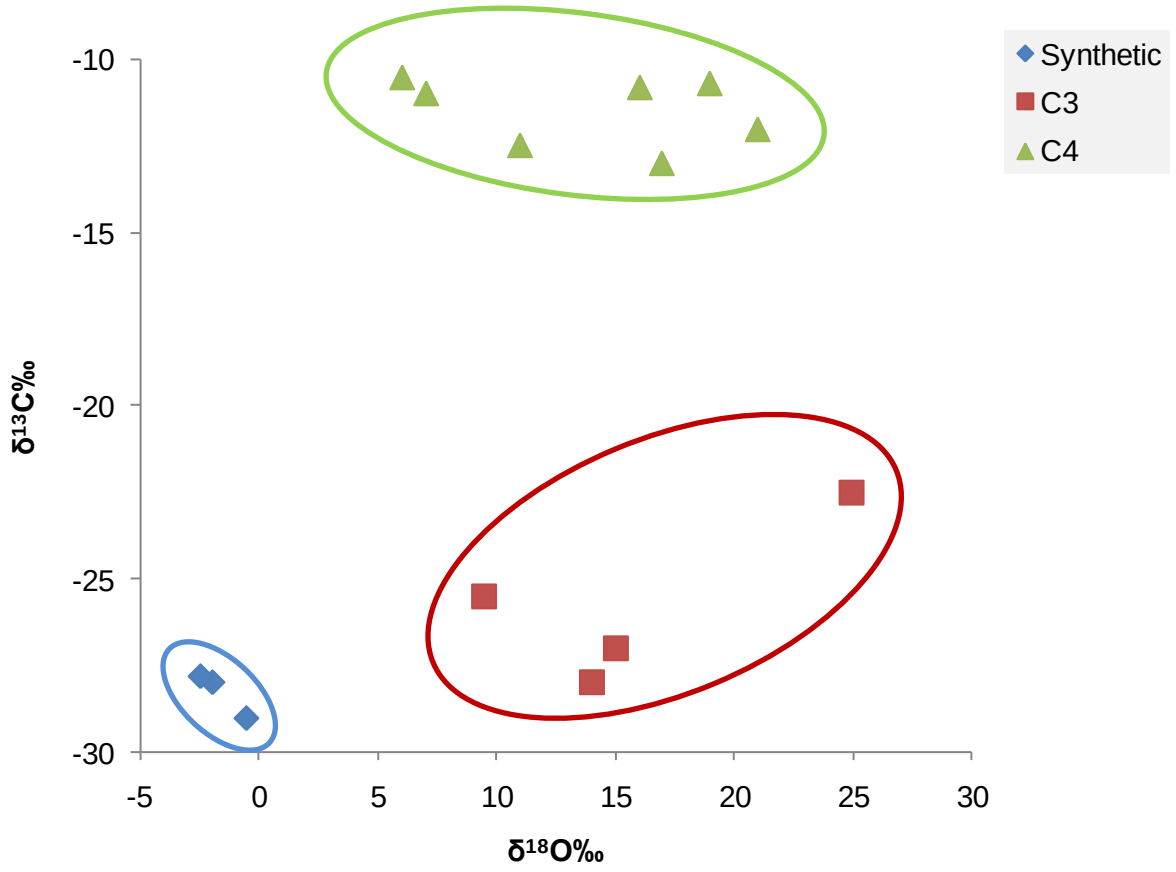

Figure 3.10 The comparison of stable isotope ratios of carbon and oxygen for ethanol samples of different origins. The diagram is a simplification of an equivalent graph presented elsewhere [Ishida-Fujii 2005].

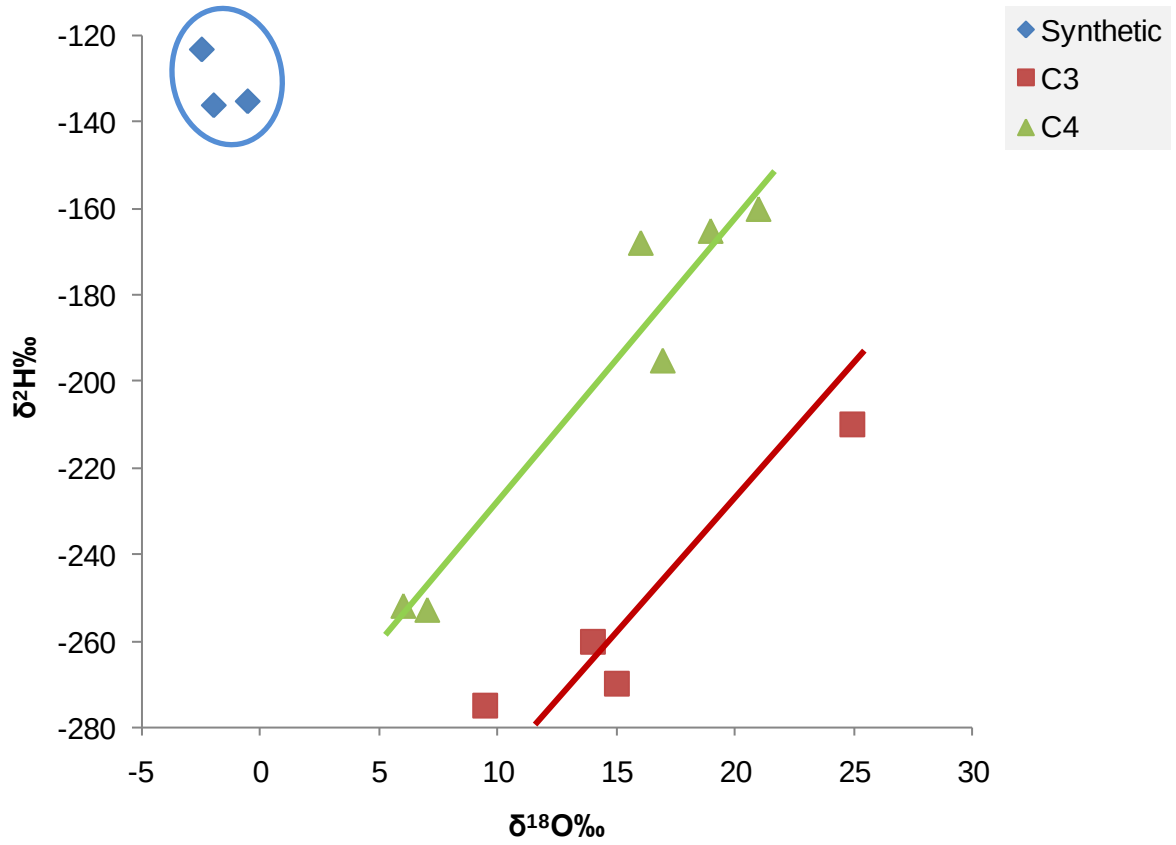

Figure 3.11 The comparison of stable isotope ratios of hydrogen and oxygen for ethanol samples of different origins. The diagram is a simplification of an equivalent graph presented elsewhere [Ishida-Fujii 2005].

Similarly, the origin of acetic acid in vinegars has also been assigned through the analysis of carbon and hydrogen isotopes [Hattori 2010]. The use of GC-IRMS was capable for this purpose. Although no synthetic acetic acid was discussed in this article, it could be that some overlap in the δ^2H hydrogen isotopic abundances between fossil feedstocks and bio-acetic acid would exist [Sowers 2006]. Without the measurement of oxygen stable isotope ratios (absent from this study) the results are not conclusive. The carbon stable isotope analysis reiterates the indistinguishable $\delta^{13}C$ values of C_3 plants and fossil based chemicals.

The use of stable isotopes to determine the farming process used to cultivate biomass has also proven to be successful [Laursen 2013]. Organic farming methods forsake the benefits of artificial fertiliser, which can be confirmed with stable isotope analysis. Hydrogen and nitrogen isotopic ratios were particularly useful in confirming the origin of the biomass, especially $^{15}N/^{14}N$ ratios. Furthermore, the oxygen content of nitrate fertiliser is dependent on its origin, be it manure or a synthetic chemical. Together a region of conventionally farmed biomass can identified graphically (**Figure 3.12**). When the method of farming is not a priority, such as in the certification of downstream bio-based products, the source of plant nutrients would seem to introduce a large variability in the ranges of a $\delta^{15}N$ observed. Ultimately

all synthetic and biological sources of nitrogen containing compounds are based on atmospheric nitrogen, and so maybe $\delta^{15}N$ is redundant as a means of identifying the biogenic atoms within chemical products.

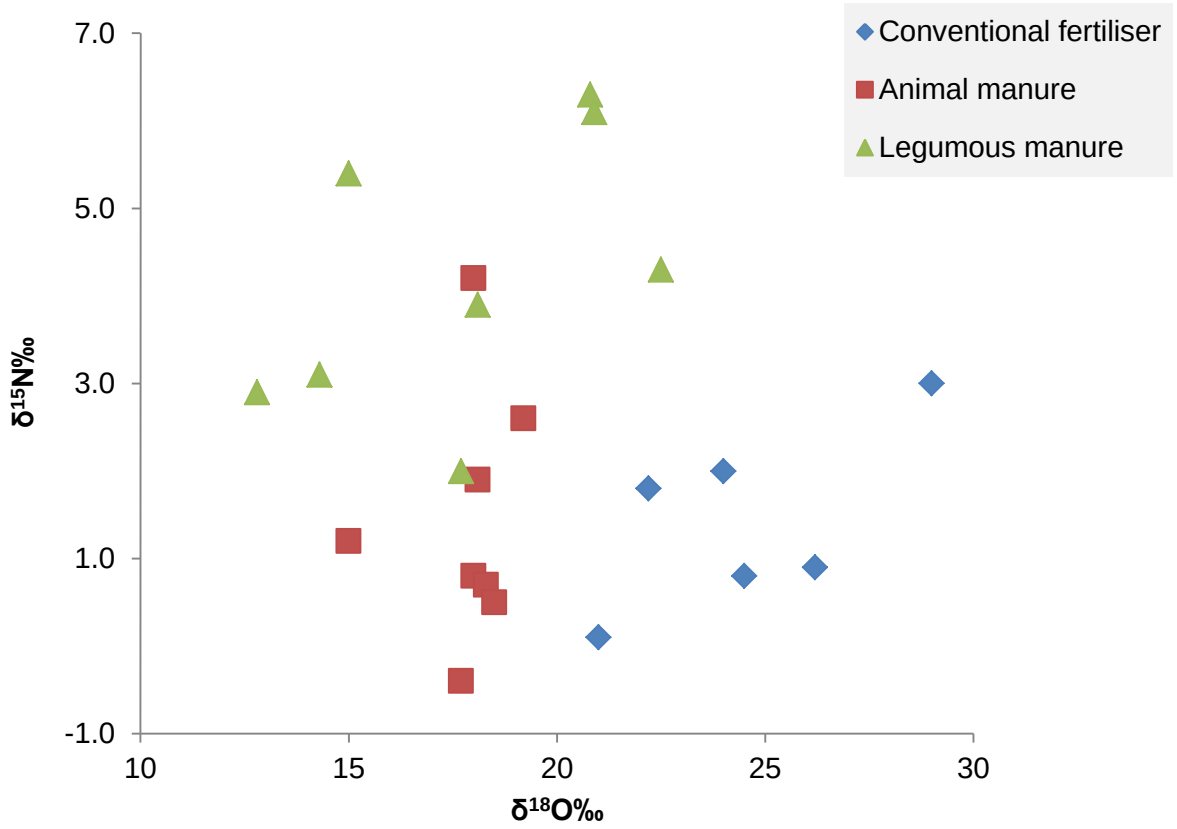

Figure 3.12 The comparison of stable isotope ratios of nitrogen in potatoes and the oxygen of the nitrates which which they were fertilised. The diagram is a simplification of an equivalent graph presented elsewhere [Laursen 2013].

Nine European laboratories have independently tested samples of cellulose to compare the reproducibility of their experiments [Boettger 2007]. Across the test centres results were generally within the expected IRMS precision limits they quote as ±0.2‰ for $\delta^{13}C$ and ±0.3‰ for $\delta^{18}O$. These error margins are more generous than suggested elsewhere [Brodie 2011]. Out of this study came the recommendation that no organic solvent washes should be used in sample preparation, and the measurement of oxygen isotopes must be performed under an argon hood. Oxygen was the stable isotope measurement most susceptible to deviation through experimental error. The issue of precision in oxygen stable isotope analysis also arises in other studies [Suzuki 2012]. The N_2 peak in EA-IRMS may overlap and contaminate the CO peak, which seems to be a common issue in this field. As such a longer column is suggested to improve resolution.

The inter-laboratory study also concurred that hydroxyl hydrogen is readily exchanged in cellulose, but carbon bound hydrogen atoms are not [Boettger 2007]. Nitration of cellulose (*i.e*. to make the infamous explosive) removes the labile exchangeable hydrogen atoms, but in doing so chemically alters the sample. This is only applicable to pure cellulose, and might not be very appropriate for processed bio-based product analysis in general. However, it does highlight that bio-based product manufacturers need to be aware of the implications of using polysaccharides in their supply chain and how these materials are used and processed.

3.5.2 The impact of pre-treatment

There are many instances reported in the scientific literature where pre-treatment measures are known to change the perceived stable isotope ratios of a sample. Usually the acid washing used to remove inorganic carbon is the cause of these errors [Brodie 2011]. Other issues arising from different pre-treatments and apparatus errors are also known, and will be explained subsequently. By understanding these effects, the negative impact they create on the isotopic analysis can be minimised. Generally the authors of work describing the isotopic fractionation falsely created by pre-treatments suggest the error is significant, but because of the large ranges of stable isotope ratios associated to elements of different origins (*e.g*. $^{13}C/^{12}C$ of C_4 plant metabolites) the error is rarely able to create new confusion over the source of the atoms that was not already an issue.

Acid treatment of organic matter samples prior to $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope analysis has been shown to introduce errors far in excess of the accepted instrument precision [Brodie 2011]. Acidification can be performed with the addition of 5% or 20% HCl in a 200:1 ratio (v/w) of acid to sample and left overnight. Then the sample is washed with water and dried in an oven at 323 K. Ineffective carbonate removal was blamed for elevated δ values, but that can only account for inaccuracies in carbon stable isotope analysis, and only when carbon and nitrogen isotopes are analysed in tandem is this a problem. It is possible to analyse nitrogen stable isotope ratios separate of carbon and other elements, in which case acid treatment is not necessary. It is preferable to analyse several of the elements in a single run for reasons of increased productivity, but it is not absolutely necessary. Nitrogen may be present as nitrates in biological material but if it is acceptable to ignore the influence of these salts no pre-treatment is usually applied when determining $^{15}N/^{14}N$ isotopic ratios. Sometimes the presence of nitrates and ammonium salts cannot be ignored for accurate analysis. The difficulty in converting nitrogen containing salts to nitrogen gas is to blame, but can be resolved under anaerobic conditions [Gentile 2013]. The cause of the error in $\delta^{15}N$ was attributed to the acid treatment and water washing inducing a loss of fine powdery material from the heterogeneous sample, obviously of a different original source than the remainder of the sample. This result is unsatisfactory but not a concern to robust formulated products, plastic composites, and other bio-based products. Material lost through rinsing was also observed in other research, and the removal of the washing stage post-acid treatment recom-

mended in order to avoid unnecessary errors [Guerin 2013, Søreide 2006]. An acid pre-treatment without a subsequent water wash allowed for accurate measurement of nitrogen isotopes [Guerin 2013]. Carbon analysis was still negatively affected however the acid pre-treatment was conducted. The authors concede that the removal of the carbonate was necessary for meaningful carbon stable isotope analysis and so it seems that a managed approach to acid treatment is the only viable way to proceed.

A fuller assessment of acid pre-treatments on hydrogen, oxygen, and sulphur stable isotope ratios has been performed [Ryba 2002]. Of the different methods of acidifying samples available, direct introduction of hydrochloric acid was deemed the most effective. However all isotopic ratios are subject to change upon acid pre-treatment and so it was recommended that only for separate carbon stable isotope analysis should acid washing be performed. The susceptability of oxygen stable isotope analysis of skeletal remains to error has also been investigated [Grottoli 2005, Wierzbowski 2007]. For the analysis of inorganic materials, bleach or peroxides are used to remove traces of organic content. This pre-treatment was found to be unnecessary and introduced unpredictable errors. Preservatives and stains can have small effects on oxygen stable isotopic ratios when part of sample preparation [Serrano 2008]. Hydrogen isotopes appear in most cases to be quite resilient to pre-treatment errors [van de Velde 2013].

Acid pre-treatments react with any carbonate salts present in the sample, producing carbon dioxide. If hydrochloric acid is used to treat calcium carbonate then a by-product of calcium chloride remains in the sample. As an involatile solid this is irrelevant to compound specific IRMS (which passes the sample through a preliminary GC separation), as would be the calcium carbonate anyway. In plastic composite products containing inorganic binders such as calcium carbonate, as an integral part of the article it too could be analysed and not removed with an acid wash just for convenience. It may be more helpful to only analyse the organic portion of the sample to work out total bio-based content. Analysis with and without acid washing and other optional pre-treatments is also possible for comparison.

For carbon, hydrogen, nitrogen, oxygen, and sulphur stable isotope analysis the decomposition of calcium carbonate to calcium chloride is not problematic. This acid wash pre-treatment is for biological samples for which only the organic material origin is of interest, and inorganic carbon interferes with the measurement. Whether it will still be of broad interest for bio-based products is not clear. Instead, implementation on a case by case basis may be preferable.

The analysis of chlorine isotopic abundance may be helpful for certain bio-based products. It is likely that many surfactants will possess chloride counter-ions, but because of concerns with chlorinated solvents only inorganic sources of chlorine are likely to be present in bio-based composite materials. If bio-based PVC becomes a viable and desirebale material then the stable isotope analysis of chlorine may become more relevant in the context of bio-based products. Presently this analysis of chlorine isotopic abundance is less routine, but

possible. A further reason to discourage considering $^{37}Cl/^{35}Cl$ isotopic ratio analysis is that sulphur and fluorine suppress the ionisation required to measure the isotope mass spectrometry of chlorine [Xiao 2007]. To help avoid this, a pre-treatment with barium carbonate is required, creating a barium sulphate precipitate that is filtered off. All the samples in this case study were water, and this precipitation technique is only applicable to aqueous formulations.

The simple processes of drying and freezing then thawing of samples have been scrutinised in order to clarify their influence on stable isotope ratios [de Lecea 2011]. It seems that oven drying and freeze drying are perfectly sound procedures and do not change the isotopic abundances of the sample. The only issue that could be found was with the freezing and thawing of biological samples, when cells structure is ruptured and intracellular material lost. This is unlikely to be an issue for bio-based products, which are unlikely to require cold storage, or for the most part comprised of delicate cellular material.

An elaborate preparation method for cellulose for isotopic abundance analysis has been developed with the intention of separating starch from plants [Wanek 2001]. Although it may not be directly relevant to the sample preparation of bio-based products prior to stable isotope analysis, it does show that the preparation of a biomass feedstock through solvent extraction and hydrolysis reactions will have a modifying effect on $\delta^{13}C$ of up to 2‰. This article provides an indication of the limitations of stable isotope analysis stemming from the wide variability of the results obtained. Prior to the conversion of biomass into commodity chemicals for further processing to make bio-based products, the conditions under which the biomass is grown may abnormally affect the observed stable isotope ratios. If a large number of birds are present then the nitrogen delivered by their droppings will change the ^{15}N isotopic abundance of the biomass [Caceres 2011]. A similar effect is known for atmospheric sulphur pollution [Winner 1978], and here when solvent extraction and hydrolysis reactions are applied stable isotope ratios are modified also [Wanek 2001].

3.5.3 Test laboratory implementation of pre-treatment

The pre-treatments practiced by a radiocarbon dating lab, the *Oxford Radiocarbon Accelerator Unit*, for ^{14}C AMS analysis and associated stable isotope analysis have been disclosed [Brock 2010]. The technique practiced in this lab (and others) is EA-IRMS, in which a 50:1 split of combusted material is established to retain a large portion of the carbon dioxide sample for graphitisation prior to AMS. Carbon and nitrogen stable isotope ratios are analysed. Although many of the pre-treatments are specific to archaeological samples, the principles of acid washing and freeze drying are of interest. The following is adapted from the literature produced by the *Oxford Radiocarbon Accelerator Unit* [Brock 2010]. Note that liquid samples are analysed directly without pre-treatment. This is applicable to bio-based lubricants and solvents. Gaseous samples are analysed with the same EA-IRMS system used for solids and liquids. Solids are discussed below to a level of detail absent from standards.

Carbonate samples such as shells and cremated bones are treated in a reaction with phosphoric acid under vacuum. Whereas acid washes are used to remove carbonates, base washes can be useful in archaeological samples to remove trapped carbon dioxide gas from the atmosphere, but may not be relevant to bio-based product testing. Each acid or base wash is followed by 3 rinses with ultrapure deionized water, although the risks of water washing have already been described. After each rinse, samples are separated by filtration, centrifugation and decanting. After the final wash, samples are freeze-dried prior to stable isotope analysis. This sample pre-treatment is performed manually in glass test tubes or centrifuge tubes. All glassware used is heated to 772 K for 3 hours before use to burn away any organic contaminants. Conducting manual pre-treatments such as these permits sample monitoring on an individual basis. Automated systems will not recognise inconsistencies, and for that reason have been abandoned by the *Oxford Radiocarbon Accelerator Unit*.

In the presence of chemical contaminants a preceding solvent extraction is applied before acid washing. Acetone followed by methanol followed by chloroform is the default process which is finished with air drying. Soxhlet extraction is also used for the removal of contaminants. The stable isotopic analysis result is scrutinised quite carefully for evidence of remaining contamination. The use of solvent extraction for fractionation has been investigated as part of another **KBBPPS deliverable D4.2** (internal report, not publicly available) but the risk of extra contamination and incomplete separations means that this is not generally recommended unless the supplier of the sample can describe a suitable procedure or supply the individual components of a bio-based product for separate testing.

After all the chemical pre-treatments, samples are frozen at 255 K and dried using a freeze dryer over 12 hours. The pressure required of 10^{-1} mbar creates a sample dry enough to permit combustion for stable isotope analysis and graphitisation for AMS. Samples are placed in tin vessels, and their carbon and nitrogen stable isotopic ratios analysed. The *Oxford Radiocarbon Accelerator Unit* use a *Carlo-Erba NA 2000* combustion elemental analyser coupled to a *Sercon 20/20* gas source isotope ratio mass spectrometer. A helium carrier gas stream of 100 mL *per* minute is used throughout. Upon combustion water is removed *via* a chemical trap to avoid protonation of carbon dioxide, which would result in $^{1}H^{12}C^{16}O_2$, which is isobaric with $^{13}C^{16}O_2$. The carbon dioxide and nitrogen gases are separated with a GC column packed with a carbon based stationary phase. Samples are admitted into the mass spectrometer *via* the aforementioned 50:1 splitter.

These long established pre-treatments, adhering to standard test methodologies where available, seem to have been refined to provide a good balance between precision and utility. Acid washing for carbonate removal will only be applicable to a small number of bio-based products. Freeze drying will be very useful for analysis across a much larger number of bio-based products, and is actually already used in methodologies developed for the preparation of samples for AMS ^{14}C radio-dating. Dry liquid samples are not subjected to any pre-treatment. It would be expected that surfactant formulations (solids dissolved in water) would be freeze dried. The remainder of the sample preparation process (combustion and

gas separation) is automated, which not only simplifies the procedure, but helps maintain reproducibility. In overview, it would appear that the principles of sample preparation needed for stable isotope analysis are well developed in a practical sense, if not specifically tailored to methods for the total bio-based content determination of bio-based products in a standardised manner. Whether or not the stable isotope analysis that follows these pre-treatments is effective in establishing bio-based content measurements is briefly addressed in the final conclusion.

4 Total biomass content determination on the basis of molecular composition

4.1 Defining biomass in terms of molecular composition

There is extensive work being conducted using analysis derived from stable isotope ratios for sample dating, source confirmation, and farming technique verification as explored in *section 3.5*. This reduces the sample to its constituent elements to derive the analysis. However, with the unique molecular structures produced by nature, from polymers such as lignin and cellulose to enantiomeric molecules such as *R*-(+)-limonene, it may be possible to identify these compounds in bio-based products, and develop a basis of bio-based content determination without resorting to elemental and isotopic analysis.

Of course the final bio-based product may have no molecular similarity to the precursor biomass, in which case only isotopic analysis will be suitable. But in some instances composite materials may contain large amounts of processed wood, plasticised starch, biosilicate adhesives, or secondary metabolites such as essential oils for example. Forms of chemical analysis to identify these ingredients must be supplemented with the reasonably safe assumption that certain chemicals (*e.g.* carbohydrate) are exclusively sourced from biomass.

Beyond the primary chemical structure of a biomass derived molecule, chirality is another indicator of product origin. Enantioselective synthesis is possible for the production of fine chemicals but will be considered as prohibitively expensive for commodity compounds such as the limonene found in consumer cleaning products. The *R*-(+)-limonene enantiomer is present in oranges and is a significant source of this compound [Clark 2012, Clark 2013, Pfaltzgraff 2013]. Other plant species, pine being one of them, express the other enantiomer and racemic limonene can be manufactured from the more readily available monoterpene α-pinene (**Figure 4.1**) [Thomas 1989]. This racemic synthetic product is bio-derived too, and so the formal identification of limonene in a formulation cannot be used to infer that it is necessarily bio-based or otherwise only the basis of chirality.

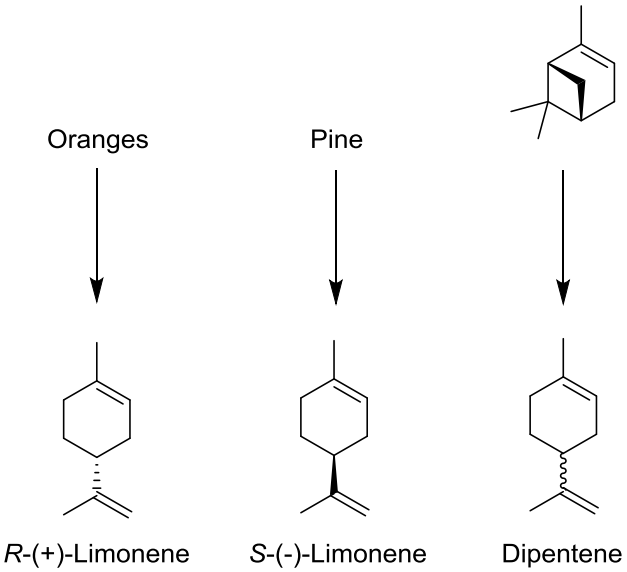

Figure 4.1 Sources of limonene.

The feedstock options for limonene production (and extraction) originate in biomass, and so in this instance chirality is redundant. It could be possible that synthetic isoprene could be used to make limonene and so it would be unwise to assume chemicals are automatically bio-based from examining their structure except in those instances documented previously (carbohydrate, lignin, *etc*.). It certainly is not a reliable or broadly applicable approach. This method of chemical identification becomes even more complicated for processed synthetic molecules. The fatty acid moiety of triglycerides can be converted into surfactants such as sodium dodecyl sulphate (**Figure 3.1**), but alternatively the dodecanol intermediate can be made by the polymerisation of ethylene (**Figure 4.2**) [Weissermel 1993]. Because ethylene can be bio-based [Braskem 2013], but is usually petroleum derived, identification of the final product or even the method of production does not necessarily help identify the source of the feedstock.

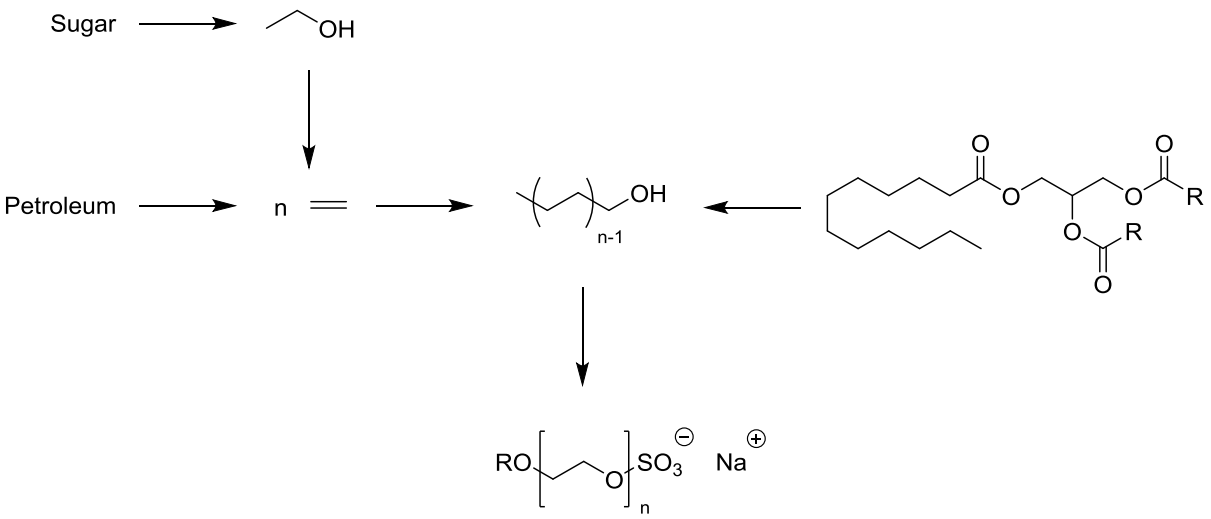

Figure 4.2 The simplified synthetic route to sodium laureth sulpahte from ethylene (using Ziegler-Natta polymerisation) or from the hydrolysis and hydrogenation of vegetable oils.

Ultimately chromatographic separation coupled with spectrometric analysis may be helpful for the verification of claims made by the chemical supplier, but not definitive in its own right. The nature of the product is important in this respect and this will probably not be broadly applicable across different product groups. However for some bio-based products, such as starch-based plastics or aqueous cleaning formulations, a method could be developed for the identification and quantification of certain components, such as starch or limonene. Spectroscopy can be used for this, and methods of analysis are presented in the following section prior to the relevant standards.

4.2 Complementary analysis techniques

4.2.1 Infra-red spectroscopy

For macromolecules, where solubility for certain methods of analysis is not possible, infra-red spectroscopy (IR) is a useful technique. Infra-red spectroscopy is applicable not only to solids and solutions, but gases as well. For example, gas phase IR is described in the project report **KBBPPS deliverable D4.1** for combustion products. This is a valuable attribute if it is to be incorporated into a horizontal methodology for bio-based product analysis, with a number of other techniques not so easily applicable across all product groups and states of matter.

The specific role of IR spectroscopy is the identification of functional groups based on the energy of the vibrational and complimentary motions of their chemical bonds. Infra-red light is passed through the sample and energy absorbed when it corresponds to the vibrational frequency of a covalent bond present in the molecule. Each absorbance is characteristic of certain bond motion; for example the carbonyl stretch resides at a wavenumber frequency of 1500-1800 cm^{-1}. Large databanks of IR spectra exist as reference material, which is the only way of equating spectra to a molecule with certainty, and even so definitive confirmation of molecular structure is tentative with IR spectroscopy alone. It is the functional groups that are identified based on their characteristic energy absorbances, not the entire molecular structure in full.

For the identification of substances such as lignin or starch, the comparison of spectra to standard reference materials will be important. Sample preparation is limited, only drying of the sample is necessary to suppress signals caused by water. Otherwise the process is quick and facile to accomplish.

4.2.2 Nuclear magnetic resonance spectroscopy

The environments of nuclei in a compound can be identified *via* the electron deshielding effects examined by nuclear magnetic resonance (NMR) spectroscopy. Unlike IR spectroscopy, a near complete understanding of the molecular composition of a compound can be attained through a combination of NMR experiments. The principle is based on the magnetic moments of nuclei interacting with an external magnetic field [Harris 1986]. The effect of neighbouring electrons changes the apparent strength of the applied magnetic field perceived by each nucleus of the molecule, and accordingly the energy (and therefore the frequency) of the transition between a nuclear spin aligned and opposed to the applied field changes. This is the basis of the assignment of chemical environments by NMR spectroscopy. Even complex macromolecules can be examined, and solid state NMR can provide an understanding of solid materials. The integrals of ^{1}H-NMR signals are proportional to the

number of nuclei creating that signal which is another important tool for chemical identification, as are the shapes of these signals.

Disadvantages of NMR spectroscopy include the difficulty of analysing mixtures of compounds. Signal overlap is an issue in complex formulations, which can also occur when one species is in a large excess compared to the others, perhaps water, creating a broad signal. If one signal is much stronger than the others then the resolution of the spectrum is hindered. Another disadvantage is the lower sensitivity of NMR spectroscopy compared to some other techniques. Even so, less than 100 mg of sample is often enough for the ^{13}C NMR spectroscopy of pure compounds. The sample is dissolved in a small amount of deuterated solvent. For formulations with many ingredients in low concentrations more sample may be required. In less complex systems, it will be possible to identify specific compounds and their relative amounts, but unfortunately these systems may be rare across the prominent bio-based products.

4.2.3 Electron spin resonance spectroscopy

Electron spin resonance (ESR) spectroscopy is equivalent to NMR spectroscopy, but whereas the latter concerns the spin of atomic nuclei, the basis of ESR spectroscopy is the spin transition of electrons [Sunandana 1998]. The electrons must be unpaired, thus this technique is suitable for the study of paramagnetic substances only. As a result ESR spectroscopy is less versatile than NMR spectroscopy, but its specificity can be put to use in the identification of radicals (that may be generated on purpose for analysis) on a quantitative basis.

4.2.4 Mass spectrometry

Mass spectrometry is routinely used to determine the mass of molecules, and not just the isotopic composition of elements within molecules as reviewed thus far [de Hoffmann 2007]. The speed and direction of the ions in the apparatus is proportional to their interaction with an electric field and a magnetic field respectively. This interaction is dependent on the mass-charge ratio of the ion which is the origin of the separation in the apparatus. These ions may have a mass representative of the original molecule (parent ions), or harsher ionisation techniques may produce charged molecular fragments. Sample compounds are ionised and after being accelerated by a magnetic field, detected from the charge they impart onto a contact surface.

Mass spectrometry for molecular structure analysis easily translates to the identification of bio-based products. The sample is typically dissolved in a solvent prior to analysis. For mixtures, the resulting mass spectrometry data will be harder to intepret. Soft ionisation techniques can be used so only parent ions are present for intepretation, which will help dis-

cern the number of components. Better still the technique can be hyphenated with a form of chromatography to separate the mixture prior to analysis. Surfactants are incompatible with regular mass spectrometers, as are some common inorganic ions. With the exception of some perfluorinated examples, ionic surfactants suppress ionisation and they may potentially cause contamination of the mass spectrometer [Ishihama 2000]. Alternative methods of compositional analysis must be used instead such as elemental analysis.

4.2.5 Chromatography

The most routine and widely available of the analytical chromatography techniques is high performance liquid chromatography (HPLC) [Hamilton 1982]. With HPLC the sample is separated on the basis of the polarity of each component as it interacts with a stationary phase. Most often the detection is conducted by UV analysis. For components lacking a chromophore light scattering techniques can be used to register the presence of a compound eluting from the column. Preparative HPLC does exist but is less common. Instead it is more common to run flash column chromatography for this purpose.

The scope of HPLC is broad, and the technique has several variants. Originally the process was normal phase, meaning that silica was used as a polar absorbent stationary phase and a low polarity solvent was used as a mobile phase. This is the same as typical column chromatography used for gravimetric analysis. Modern HPLC works in reverse phase to improve consistency and reproducibility. The stationary phase is more hydrophobic (silica functionalised with hydrocarbon pendant groups) and the solvent used for the mobile phase is usually aqueous acetonitrile. Primarily the choice of stationary phase is dictated by the substrate. Surfactants must be analysed with silica functionalised with ionic groups, and macromolecules require a stationary phase with a different pore size distribution to those suitable for smaller molecules.

Gas chromatography is an analytical technique very similar to HPLC, except that the sample is separated into its constituent components based on their relative volatility as well as their affinity for the stationary phase in the absence of a solvent. This process forms the first part of compound specific IRMS. Heating of the sample (dissolved in a solvent) is ramped from the ambient temperature to 573 K, or hotter sometimes if needed [Grob 1985]. As the components parts of the sample evaporate they pass through the separation column as with other types of chromatography. Compounds with similar boiling points (such as terpenes) can be separated if they are of different polarities. Compounds with similar polarities (such as the triglycerides in a vegetable oil) can be separated on the basis of their volatility. The technique benefits from little sample preparation (dissolve in a solvent and filter) and reasonably quick separation and analysis time.

Very volatile components such as solvents and thermally unstable compounds are best analysed by different methods to GC. Weak ionisation potential is also not very helpful

as detection is often supplemented by mass spectrometry. Flame ionisation detection (FID) is a more robust alternative, measuring concentrations of compounds in the gas phase, but the characterisation of each fraction would need to be supplemented with the use of a standard reference material and further spectroscopy.

Whether HPLC, gas chromatography, or other techniques such as supercritical fluid chromatography is used, a hyphenated technique in which a mass spectrometer provides further analysis on the sample would be beneficial. This of course replicates the compound specific IRMS approach explained previously. A FID detector permits a quantitative analysis of the sample molecular composition, but lacks the definitive molecular structure information gained through mass spectrometry. Running both types of analysis would be preferable when possible. Research is currently being conducted on GC-AMS hyphenated systems, which could develop into a highly useful technique for the biogenic analysis of formulations using radioisotope ratios [ORAU 2013].

4.2.6 Enantiomeric analysis

Chiral compounds can rotate plane polarised light, with enantiomers operating as opposites in this respect [Landolt 1902]. An enantiomeric excess of a compound can be determined based on an optically pure single enantiomer standard. Racemic mixtures do not rotate plane polarised light. This analysis is perfectly legitimate for pure samples, but mixtures and formulated end products cannot be used. Sometimes the crystalline form of a substance will be optically active and the amorphous form not [Landolt 1902]. Sometimes the opposite is true; crystalline sucrose is not applicable to this analysis, but amorphous samples are [Mackenzie 1913].

Chromatography based on chiral column separation is a much more obvious technique for the analysis of bio-based products. As a form of liquid chromatography, it is not applicable to insoluble materials. Plastics and other polymeric or macromolecular materials, for the most part, may not need to be analysed for enantiometric excess anyway. Polyethylene made from bio-ethanol for instance is achiral. Plasticised starch containing materials could be analysed more effectively by IR spectroscopy than by basing an analysis on chirality. A notable instance of a chiral and bio-based polymer is poly(lactic acid). The *L*-lactide is the most prevalent version, but some biological organisims produce the other stereoisomer of lactic acid [Chen 2012, Singhvi 2013], and polymer blends and co-polymers of the two monomers are available. The chemical synthesis of lactic acid from acetaldehyde and hydrogen cyanide produces a racemic product. Differentiation between these product types could help assist the determination of bio-based content, but only if the *L*-lactide remained the predominant form produced from biomass (by fermentation) and if no racemisation occurs during polymer production and processing. An equal mixture of the two stereoisomers would not rotate plane polarised light and could be mistaken for the racemic fossil based product, complicating the concept of biogenic analysis by chirality.

4.3 Sample preparation and standards for compositional analysis

4.3.1 Overview of spectroscopic and chromatographic standard test methods

The analytical techniques discussed thus far for the identification of the different molecules in a bio-based product are widely used, and have standardised procedures associated with them for specific applications (**Table 4.1**). Prior to performing any of these spectroscopic methods, the required sample preparation is no more complex than that already discussed at length with respect to relevant standard test methodologies for elemental and isotopic analysis. In fact, many of those standards cited as containing suitable sample preparation procedures for isotopic analysis, were actually general purpose methods, applicable to an assortment of possible analyses. Instead of just reiterating the necessary measures for the homogenisation and drying of samples, further standards that could reveal the biogenic origin of chemicals on a molecular basis have been tabulated below, followed by general notes on the most promising examples.

Table 4.1 Relevant standardised procedures for the compositional analysis of bio-based products.

Class	Name	Technique
Chromatography		
	ASTM D2245	Standard Test Method for Identification of Oils and Oil Acid in Solvent-Reducible Paints
	ASTM D2549	Standard Test Method for Separation of Representative Aromatics and Nonaromatics Fractions of High-Boiling Oils by Elution Chromatography
	ASTM D2743	Standard Practices for Uniformity of Traffic Paint Vehicle Solids by Spectroscopy and Gas Chromatography
	ASTM D2887	Standard Test Method for Boiling Range Distribution of Petroleum Fractions by Gas Chromatography
	ASTM D3452	Standard Practice for Rubber - Identification by Pyrolysis-Gas Chromatography
	ASTM D3465	Standard Test Method for Purity of Monomeric Plasticizers by Gas Chromatography
	ASTM D4424	Standard Test Method for Butylene Analysis by Gas Chromatography
	ASTM D4626	Standard Practice for Calculation of Gas Chromatographic Response Factors
	ASTM D4827	Standard Test Method for Determining the Unreacted Monomer Content of Latexes Using Capillary Column Gas Chromatography
	ASTM D5599	Standard Test Method for Determination of Oxygenates in Gasoline by Gas Chromatography and Oxygen Selective Flame Ionization Detection
	ASTM D5623	Standard Test Method for Sulfur Compounds in Light Petroleum Liquids by Gas Chromatography and Sulfur Selective Detection
	ASTM D5769	Standard Test Method for Determination of Benzene, Toluene, and Total Aromatics in Finished Gasolines by Gas Chromatography/Mass Spectrometry
	ASTM D5986	Standard Test Method for Determination of Oxygenates, Benzene, Toluene, C8-C 12 Aromatics and Total Aromatics in Finished Gasoline by Gas Chromatography/Fourier Transform Infrared Spectroscopy
	ASTM D6159	Standard Test Method for Determination of Hydrocarbon Impurities in Ethylene by Gas Chromatography
	ASTM D6550	Standard Test Method for Determination of Olefin Content of Gasolines by Supercritical-Fluid Chromatography

Table 4.1 Relevant standardised procedures for the compositional analysis of bio-based products (continued).

Class	Name	Technique
Chromatography		
	ASTM D7419	Standard Test Method for Determination of Total Aromatics and Total Saturates in Lube Basestocks by High Performance Liquid Chromatography (HPLC) with Refractive Index Detection
	ASTM D7423	Standard Test Method for Determination of Oxygenates in C2, C3, C4, and C5 Hydrocarbon Matrices by Gas Chromatography and Flame Ionization Detection
	ASTM D7845	Standard Test Method for Determination of Chemical Species in Marine Fuel Oil by Multidimensional Gas Chromatography – Mass Spectrometry
	ASTM E260	Standard Practice for Packed Column Gas Chromatography
	ASTM E516	Standard Practice for Testing Thermal Conductivity Detectors Used in Gas Chromatography
	ASTM E1303	Practice for Refractive Index Detectors Used in Liquid Chromatography
	ASTM E1642	Standard Practice for General Techniques of Gas Chromatography Infrared (GC/IR) Analysis
	ASTM E2106	Standard Practice for General Techniques of Liquid Chromatography-Infrared (LC/IR) and Size Exclusion Chromatography-Infrared (SEC/IR) Analyses
	EN 1378	Foodstuffs - Determination of aspartame in table top sweetener preparations - Method by high performance liquid chromatography
	EN 12137	Fruit and vegetable juices - Determination of tartaric acid in grape juices - Method by high performance liquid chromatography
	EN 12630	Fruit and vegetable juices - Determination of glucose, fructose, sorbitol and sucrose contents - Method using high performance liquid chromatography
	EN 13273	Surface active agents - Determination of the content of non-ionic substances in anionic surface active agents by high performance liquid chromatography (HPLC)
	EN 13405	Surface active agents - Determination of dialkyl-tetralins content in linear alkylbenzene by high performance liquid chromatography (HPLC)
	EN 15360	Fertilizers - Determination of dicyandiamide - Method using high-performance liquid chromatography (HPLC)

Table 4.1 Relevant standardised procedures for the compositional analysis of bio-based products (continued).

Class	Name	Technique
Chromatography		
	EN 15911	Foodstuffs - Simultaneous determination of nine sweeteners by high performance liquid chromatography and evaporative light scattering detection
	EN 27941	Commercial propane and butane - Analysis by gas chromatography (ISO 7941:1988)
	EN ISO 5508	Animal and vegetable fats and oils - Analysis by gas chromatography of methyl esters of fatty acids (ISO 5508:1990)
	EN ISO 9936	Animal and vegetable fats and oils - Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography (ISO 9936:2006)
	EN ISO 10504	Starch derivative - Determination of the composition of glucose syrups, fructose syrups and hydrogenated glucose syrups - Method using high-performance liquid chromatography (ISO 10504:1998)
	EN ISO 11337	Plastics - Polyamides - Determination of e-caprolactam and w-laurolactam by gas chromatography (ISO 11337:2010)
	EN ISO 14939	Animal feeding stuffs - Determination of carbadox content - Method using high-performance liquid chromatography (ISO 14939:2001)
	EN ISO 17226-1	Leather - Chemical determination of formaldehyde content - Part 1: Method using high performance liquid chromatography (ISO 17226-1:2008)
	EN ISO 19739	Natural gas - Determination of sulfur compounds using gas chromatography (ISO 19739:2004)
	EN ISO 22854	Liquid petroleum products - Determination of hydrocarbon types and oxygenates in automotive-motor gasoline - Multidimensional gas chromatography method (ISO 22854:2008)
	ISO 2718	Standard layout for a method of chemical analysis by gas chromatography
	ISO 5508	Animal and vegetable fats and oils - Analysis by gas chromatography of methyl esters of fatty acids
	ISO 6225-1	Rubber, raw, natural-- Determination of castor oil content - Part 1: Determination of castor oil glyceride content by thin-layer chromatography
	ISO 6841	Surface active agents - Technical straight-chain sodium alkylbenzenesulfonates - Determination of mean relative molecular mass by gas-liquid chromatography

Table 4.1 Relevant standardised procedures for the compositional analysis of bio-based products (continued).

Class	Name	Technique
Chromatography		
	ISO 6974-3	Natural gas - Determination of composition with defined uncertainty by gas chromatography - Part 3: Determination of hydrogen, helium, oxygen, nitrogen, carbon dioxide and hydrocarbons up to C8 using two packed columns
	ISO 7358	Oils of bergamot, lemon, citron and lime, fully or partially reduced in bergapten - Determination of bergapten content by high-pressure liquid chromatography (HPLC)
	ISO 7609	Essential oils - Analysis by gas chromatography on capillary columns - General method
	ISO 8432	Essential oils - Analysis by high performance liquid chromatography - General method
	ISO 10504	Starch derivatives - Determination of the composition of glucose syrups, fructose syrups and hydrogenated glucose syrups - Method using high-performance liquid chromatography
	ISO 11337	Plastics - Polyamides - Determination of ε-caprolactam and ω-laurolactam by gas chromatography
	ISO 11401	Plastics - Phenolic resins - Separation by liquid chromatography
	ISO 12292	Instant coffee - Determination of free and total carbohydrate contents - Method using high-performance anion-exchange chromatography
	ISO 13885-1	Binders for paints and varnishes - Gel permeation chromatography (GPC) - Part 1: Tetrahydrofuran (THF) as eluent
	ISO 15302	Animal and vegetable fats and oils - Determination of benzo[a]pyrene - Reverse-phase high performance liquid chromatography method
	ISO 16931	Animal and vegetable fats and oils -- Determination of polymerized triacylglycerols by high-performance size-exclusion chromatography (HPSEC)
	ISO 17059	Oilseeds - Extraction of oil and preparation of methyl esters of triglyceride fatty acids for analysis by gas chromatography (Rapid method)
	ISO 20481	Coffee and coffee products - Determination of the caffeine content using high performance liquid chromatography (HPLC) - Reference method

Table 4.1 Relevant standardised procedures for the compositional analysis of bio-based products (continued).

Class	Name	Technique
Chromatography		
	ISO 22854	Liquid petroleum products - Determination of hydrocarbon types and oxygenates in automotive-motor gasoline - Multi-dimensional gas chromatography method
	ISO 22855	Fruit and vegetable products - Determination of benzoic acid and sorbic acid concentrations - High performance liquid chromatography method
	ISO 22972	Essential oils - Analysis by gas chromatography on chiral capillary columns - General method
Electron spin resonance spectroscopy		
	EN 1786	Foodstuffs - Detection of irradiated food containing bone - Method by ESR spectroscopy
	EN 1787	Foodstuffs - Detection of irradiated food containing cellulose by ESR spectroscopy
	EN 13708	Foodstuffs - Detection of irradiated food containing crystalline sugar by ESR spectroscopy
Infra-red spectroscopy		
	ASTM D2238	Standard Test Methods for Absorbance of Polyethylene Due to Methyl Groups at 1378 cm^{-1}
	ASTM D5576	Standard Practice for Determination of Structural Features in Polyolefins and Polyolefin Copolymers by Infrared Spectro-photometry (FT-IR)
	ASTM D5845	Standard Test Method for Determination of MTBE, ETBE, TAME, DIPE, Methanol, Ethanol and tert-Butanol in Gasoline by Infrared Spectroscopy
	ASTM D7371	Standard Test Method for Determination of Biodiesel (Fatty Acid Methyl Esters) Content in Diesel Fuel Oil Using Mid In-frared Spectroscopy (FTIR-ATR-PLS Method)
	ASTM D7399	Standard Test Method for Determination of the Amount of Polypropylene (PP) in Polypropylene/LDPE Mixtures Using Infrared Spectrophotometer (FTIR)
	ASTM D7588	Standard Guide for FT-IR Fingerprinting of a Non-Aqueous Liquid Paint as Supplied in the Manufacturer's Container
	ASTM D7806	Standard Test Method for Determination of the Fatty Acid Methyl Ester (FAME) Content of a Blend of Biodiesel and Petroleum-Based Diesel Fuel Oil Using Mid-Infrared Spec-troscopy

Table 4.1 Relevant standardised procedures for the compositional analysis of bio-based products (continued).

Class	Name	Technique
Infra-red spectroscopy		
	ASTM E168	Standard Practices for General Techniques of Infrared Quantitative Analysis
	ASTM E204	Standard Practices for Identification of Material by Infrared Absorption Spectroscopy, Using the ASTM Coded Band and Chemical Classification Index
	ASTM E1252	Standard Practice for General Techniques for Obtaining Infrared Spectra for Qualitative Analysis
	ISO 15063	Plastics - Polyols for use in the production of polyurethanes - Determination of hydroxyl number by NIR spectroscopy
	ISO 14558	Rubber - Determination of residual unsaturation of hydrogenated nitrile rubber (HNBR) by infrared spectroscopy
Mass spectrometry		
	ASTM D2425	Standard Test Method for Hydrocarbon Types in Middle Distillates by Mass Spectrometry
	ASTM D2650	Standard Test Method for Chemical Composition of Gases By Mass Spectrometry
	ASTM D3239	Standard Test Method for Aromatic Types Analysis of Gas-Oil Aromatic Fractions by High Ionizing Voltage Mass Spectrometry
Nuclear magnetic spectroscopy		
	ASTM D5017	4.3.1.1 Standard Test Method for Determination of Linear Low Density Polyethylene (LLDPE) Composition by Carbon-13 Nuclear Magnetic Resonance
	ASTM D5292	Standard Test Method for Aromatic Carbon Contents of Hydrocarbon Oils by High Resolution Nuclear Magnetic Resonance Spectroscopy
	ASTM D7171	Standard Test Method for Hydrogen Content of Middle Distillate Petroleum Products by Low-Resolution Pulsed Nuclear Magnetic Resonance Spectroscopy
	ISO 8292	Animal and vegetable fats and oils - Determination of solid fat content by pulsed NMR - Part 1: Direct method
	ISO 10632	Oilseed residues - Simultaneous determination of oil and water contents - Method using pulsed nuclear magnetic resonance spectroscopy
	ISO 11543	Modified starch - Determination of hydroxypropyl content - Method using proton nuclear magnetic resonance (NMR) spectrometry

Table 4.1 Relevant standardised procedures for the compositional analysis of bio-based products (continued).

Class	Name	Technique
Water content		
	ASTM D4006	Standard Test Method for Water in Crude Oil by Distillation
	ASTM D5460	Standard Test Method for Rubber Compounding Materials—Water in Rubber Additives
	ASTM D6304	Standard Test Method for Determination of Water in Petroleum Products, Lubricating Oils, and Additives by Coulometric Karl Fischer Titration
	ASTM E203	Standard Test Method for Water Using Volumetric Karl Fischer Titration
	ASTM E1064	Standard Test Method for Water in Organic Liquids by Coulometric Karl Fischer Titration
	ISO 15512	Plastics - Determination of water content

Most spectroscopic methods of analysis are hindered by high water content, and so moisture removal will be crucial if any value is to be gleaned from this approach. This is covered by **ASTM E1757** and other standards. Analysis by a chromatographic method requires that the sample is dissolved in a solvent, as does NMR spectroscopy. Generally chromatographic standards are based on GC. Although prevalent they are all highly specific to a certain product or a class of compounds, often a petroleum distillation cut or a foodstuff. This is a large disadvantage when compared to the use of isotope ratios, which by combusting the sample is equally application to products of all varieties.

4.3.2 European standards

EN 12630 Fruit and vegetable juices - Determination of glucose, fructose, sorbitol and sucrose contents - Method using high performance liquid chromatography

The application of HPLC for the analysis of sugars (by their refractive index) in fruit juice is standardised as defined by **EN 12630**. Solids are removed by centrifugation. High performance liquid chromatography is able to separate fructose, glucose, sorbitol, and sucrose on an analytical scale with a mobile phase buffered with ethylenediaminetetraacetic acid (EDTA). The calculation for sugar concentration is described in **EN 12630** and requires the use of standards to calibrate the analysis. Repeatability and reproducibility has been described as being of sufficient accuracy.

This specialised standard test method demonstrates the principle that analytical chromatography can be used to identify the components or a mixture and quantify the composition of bio-based products. This will be applicable to certain solvents and lubricants. Both

can alternatively be analysed by GC. Liquid chromatography under specific conditions can also be used to separate surfactants [Nesterenko 2006].

EN 1787 Foodstuffs - Detection of irradiated food containing cellulose by ESR spectroscopy

Strawberry seeds, pistachio nuts, and powdered paprika can be analysed for radiation treated cellulose content with ESR spectroscopy. Freeze drying is recommended, but size reduction by grinding is not for the ESR signal strength is diminished. Overall, inter-laboratory data is tainted with inconsistencies; both false positives and false negatives have been recorded (up to 20% of the total number of samples for some product types). This type of analysis is only applicable to a small range of chemicals anyway, and treatment of the sample prior to analysis (*i.e*. irradiation) dictates the result.

4.3.3 Other standards

ISO 356 Essential oils – Preparation of test samples

If the sample is a solid, it should be melted without air being allowed to enter, otherwise some oxidation may occur. Liquid samples are then dried with magnesium sulphate or sodium sulphate (15 wt% added with stirring). To filter the dehydrating agent from the solid essential oil samples, the procedure must be conducted in an oven just above its melting point. Prolonged heating is discouraged. Only drying is stated in this standard method as a requirement for the sample preparation for oils.

ISO 8432 Essential oils - Analysis by high performance liquid chromatography - General method

A general methodology for GC analysis has been presented as **ISO 2718**: *Standard layout for a method of chemical analysis by gas chromatography*. Not much detail is provided beyond the calibration of the instrument and the way in which the analysis should be reported. The more specific **ISO 8432** gives details on the HPLC analysis of essential oils, although again only the necessary details concerning operating conditions and calibration are provided. The chromatographic analysis of essential oils requires that the individual components are sufficiently resolved, and to do so column efficiency and resolution need to meet the minimum requirements of **ISO 8432**. This does depend on the nature of the substances being separated and so a single method cannot serve all possible samples. As with the general standard that is **ISO 2718**, the subject of calibration is also addressed in **ISO 8432**. It is important that calibration involves deducing the relationship between peak area and concentration from many samples prepared at different but known concentrations for this purpose.

ISO 22972 Essential oils – Analysis by gas chromatography on chiral capillary columns – General method

Enatiomeric excess is one characteristic of many bio-based molecules that could be exploited to provide an estimate of total biomass content, **ISO 22972** being one of these. The use of chiral gas chromatography columns for the analysis of essential oils is specified by **ISO 22972**, but it is a rarity; more frequently only achiral chromatographic separation is practiced. Sample preparation is minor post-sampling, dealt with in a separate standard with an emphasis on solids (**ISO 356**). Chemical transformations performed on the oil, such as derivatisation for improved analysis, remain applicable but are not helpful from the perspective that a change in composition is created by the modification of components within the bio-based product sample. Solvent (mobile phase) selection and method optimisation generally presents a bigger issue than sample preparation. The same applies to test methods such as that described in **ISO 8432** for the analysis of essential oils by HPLC.

ISO 11543 Modified starch - Determination of hydroxypropyl content - Method using proton nuclear magnetic resonance (NMR) spectrometry

Nuclear magnetic resonance spectroscopy is a very powerful tool in the identification of chemicals, applied here in **ISO 11543** for the analysis of modified starch. The sample is sieved, and particles larger than 0.8 mm must be processed with a mill until meeting the size requirements. The standard then calls for the sample to be homogenised without further instructions. Washing of the starch material is outlined, and the solid is isolated by vaccum filtration rather than using the centrifuge technique familiar with many other standard test methods featured earlier in this report.

The sample is dissolved in a deuterated brine solution and the spectrum obtained in the usual manner without further sample preparation. A relaxation time of fifteen seconds is required between electromagnetic pulses. For the calculation of hydroxypropyl content from the NMR spectrum the dry mass of the sample used must be known. This can be obtained in accordance with **ISO 1666**. An internal standard is used, eliminating the need for a calibration.

ASTM D5017 Standard Test Method for Determination of Linear Low Density Polyethylene (LLDPE) Composition by Carbon-13 Nuclear Magnetic Resonance

The determination of the composition of a co-polymerised material will offer a meaningful insight into the total bio-based content of a synthetic product of renewable and non-renewable reactants. In the specific example addressed by **ASTM D5017**, co-polymers of ethylene and other alkenes are analysed with high temperature ^{13}C NMR spectroscopy. This analysis is limited to certain types of co-polymer. Block co-polymers for instance are not applicable. Assignment of different monomers within the sample is achieved through the comparison of the observed signals in the spectrum to reference chemical shifts presented in

ASTM D5017. No sample pre-treatment is required other than dissolution in a deuterated solvent, which makes the process attractive from a throughput efficiency perspective. Conversely this standard test method is severely curtailed by the range of suitable substrates, and ultimately is possibly too specialised to provide a meaningful contribution to the biogenic analysis of bioplastics as a whole.

ASTM D2650 Standard Test Method for Chemical Composition of Gases By Mass Spectrometry

Gases, as with all volatile species, can be analysed by GC. The tabulated data provided in **ASTM D2650** assists in the diagnosis of gaseous components in a sample. The procedure for introducing the sample to the mass spectrometer is explained on a technical level but in terms of sample preparation no extra considerations are raised. Gas mixtures are inherently homogeneous. The analysis of isomers (*e.g.* butenes) required further spectrometric techniques and so this does not constitute a complete analytical assessment. In reality the mass spectrometry of a sample and its component parts must be supplemented with other procedures to maximise its usefulness, most obviously GC coupled with mass spectrometry.

ASTM D7399 Standard Test Method for Determination of the Amount of Polypropylene (PP) in Polypropylene/LDPE Mixtures Using Infrared Spectrophotometer (FTIR)

It is possible that blends of bioplastics and conventional fossil based plastics will make up a considerable portion of the bio-based product market as a whole in the future. Therefore diagnostic checks to help identify the components of an article will prove valuable. In **ASTM D7399** the amount of polypropylene in its mixtures with polyethylene is resolved with IR spectroscopy. Polyethylene is now manufactured from biomass [Braskem 2013], and so blended plastic materials of this sort could consist of a mixture of bio-based material and petroleum derived material. Infra-red spectroscopy to determine the amount of polyethylene, used in conjuction with stable carbon isotope analysis to indicate the origin of the plastic, could be an insightful exercise because sugarcane derived products have a distinct $^{13}C/^{12}C$ ratio to fossil based products (**Table 3.3**).

No sample preparation is required, a feature that emphasises the usefulness of IR spectroscopy. The characteristic absorbance of polyethylene at 1465 cm^{-1}, and that of polypropylene at 1373 cm^{-1}, are used to assign an absorbance ratio that must be fitted to results from standard materials with a known ratio of polyethylene to polypropylene in order to assign an accurate compositional ratio.

ASTM D7806 Standard Test Method for Determination of the Fatty Acid Methyl Ester (FAME) Content of a Blend of Biodiesel and Petroleum-Based Diesel Fuel Oil Using Mid-Infrared Spectroscopy

Infra-red spectroscopy is used once again, but in **ASTM D7806** it is for the determination of esters in a blend of renewable biodiesel and conventional petroleum derived diesel. This mirrors the scope of **ASTM D7399** in which blends of two components, one possibly bio-based, are resolved by IR spectroscopy. Sampling is covered by **ASTM D4057** and **ASTM D4177**, after which no special sample preparation is required. Technicians are reminded not to expose the sample unnecessarily to excessive heat, and the infra-red transmission cell must be purged correctly to avoid contamination. Analysis of bio-diesel content is achieved through the analysis of the distinctive carbonyl stretch between 1713 cm^{-1} and 1784 cm^{-1}. This standard test method may have use in the analysis of bio-based lubricants, themselves likely to be esters.

ISO 15512 Plastics - Determination of water content

The determination of water content is very important. Perhaps chromatography and spectroscopy will be used to aid the assignment of total bio-based content, in which case water will likely form a significant proportion of the composition of some bio-based products and must be recognised. If total bio-based content is to be performed on the basis of organic content (as bio-based carbon content currently is with **ASTM D6866**) then water, along with inorganic salts *etc.*, will need subtracted form subsequent calculations. The only way to do this is to dry the sample until analysis is viable, and then determine the residual water content. Plastic products are the biggest chemical product sector, and so the water content of bioplastics will be an area of interest. Conventional plastics like polyethylene will have minimal, probably negligible water content, but plasticised starch based products are likely to contain enough water to impact the mass balance of components and therefore form a crucial part of sample preparation.

Three methods for the determination of water content in plastics are presented in **ISO 15512**. The first is a solvent extraction with methanol, verified by Karl-Fischer titration. Sample preparation involves reducing the sample to 0.001 g portions. Anhydrous methanol is added and refluxed for 3 hours under condition that forbid the introduction of atmospheric water vapour. A blank reflux is also conducted as a background check. The liberated water, now as a methanol solution, is titrated in accordance with **ISO 760**.

The second method for the determination of water content in plastics gives a greater precision (0.01%) than the first method (0.1%), and is based on drying by evaporation of the water caused by heat or a flow of gas. A coulometric Karl Fischer titrator is used to analyse the water of the sample that becomes vaporised. Heating to induce evaporation can be conducted up to 573 K.

The final water content determination methodology only works in the absence of other volatile species, for it is dependent on the pressure change created upon evaporation of the water in a vacuum. Gas chromatography must be used to check for the presence of other volatile chemicals that may interfere with this analysis. The speciality equipment required for this analysis may lead to a preference for the first two methods of water determination.

5 Conclusion

This report, *Sample Preparation Techniques For Total Biomass Content Determination*, that being deliverable 4.3 of the KBBPPS project, has been developed to provide an understanding of the current state of the art concerning the laboratory procedures implemented prior to chemical analysis that may lead to the determination of total bio-based content. This work complements a report on sample preparation (**KBBPPS deliverable D3.1**), and preceeds original research on stable isotope analysis that will not be made public. The summarises of standard test methods provided within may feed into a future report to be produced by the KBBPPS project concerning a generally applicable methodology for the sampling and measurement of bio-based carbon content (**KBBPPS deliverable D3.6**, due to be published in 2015) that is centred on ^{14}C radiocarbon analysis.

During the course of assembling this review of standards relating to sample preparations, some trends have emerged relating to the processing of potentially bio-based products. It would seem that prior to chemical analysis a sample should be dry and homogeneous. Numerous standard test methods document procedures for achieving this. It shall be assumed that as an inorganic component, water will be discounted from total bio-based content calculations, and physical removal of water prior to any analysis would be the most fitting way to discount it. For the most familar bio-based product classes (lubricants, plastics, solvents, and surfactants), drying at 378 K in an oven would seem a reasonable measure to adopt for some articles, but only if decomposition or loss of volatiles was not encouraged. The lower oven temperatures used for the drying of biomass (typically 318 K) would not be appropriate. Aqueous formulations will require freeze drying, as documented **ASTM E1757** and could be applied all across product groups except the volatile solvents or products contain volatile components such as fragrances.

Lubricants and solvents are homogeneous, as are all gaseous products that might be considered as bio-based. Most surfactant solutions are stable systems and so would also be exempt from any homogenisation process. In the first instance homogeneity must be established with proper sampling (**KBBPPS deliverable D3.1**). Then procedures for reducing the particle size of plastics and solid composite materials must retain this homogeniety while also presenting a sample correct for analysis. Such protocols have been documented repeatedly in the standards reviewed in this document. Cutting mills would seem to be appropriate for all plastics, sometimes after a preliminary freeze crushing as described in **EN 15413** in order to provide an intermediary particle size and reduce the burden on the cutting mill. If the sample is a plastic film, homogeneous in nature as opposed to a layered or multi-phase material, then cutting with scissors is satisfactory. Ultimately particle size will not effect the determina-

tion of total bio-based content, and so any cutting and crushing procedures can be used in order to reduce the minimum available sample size, but beyond that are not vital.

It is helpful to follow through the process with an example, and for this role sodium laureth sulphate can be used. This hypothetical sample preparation method can be concluded by foreseeing any potential issues with the subsequent stable isotope analysis or a complementary analysis technique. If we assume the surfactant is supplied as a 10% solution (on the basis of mass) in water, then up to 90% of the sample can be removed by freeze drying (efficiency is unlikely to be maximal). Oven drying is prominent in the sample preparation of solid biomass, but for aqueous solutions this is inefficient and prone to bumping and loss of sample. Prolonged heating may cause hydrolysis of the surfactant and degradation of the bio-based product. After freeze drying, the resultant substance would ideally be a solid. In some instances the dried material might be a firm gel, for water is often tighly bound to surfactant molecules, and this is the reason why they are supplied as aqueous solutions. Therefore water content determination should be performed according to a standard; **ASTM E203** is a suitable general purpose test method. For bio-based products such as solvents that will evaporate if oven dried or freeze dried, **ASTM E1064** provides a standard test method for the detection of trace amounts of water. After freeze drying, storage in a sealed container would be necessary for hygoscopic materials.

Once a dry sample is prepared, homgeneity is required. A single substance cannot be heterogeneous and so the sampling should not have introduced a bias. Similarly reduction of sample size and modfication of particle size should not be an issue in this respect either. The latter process should be possible with the previously described processes of freeze crushing and/or milling. As described previously, particle size is not a priority and so cutting a suitably sized portion of a solid homogeneous material by other means would be acceptable if in line with protocol.

Analysis of sodium laureth sulphate by elemental analysis should yield values close to the theoretical values presented in **Table 3.1** of 51% carbon, 9% hydrogen, 0% nitrogen, and 40% other elements if dry. Oxygen and sulphur elemental analysis can also be performed and should suggest 27% and 8% by mass of these elements respectively. A remaining 5% of the sample mass should be sodium, and might be inferred from ICP analysis or another suitable technique. The inorganic portion of the sodium laureth sulphate must be decided based on this analysis if it is to be discounted from total bio-based content analysis. It is not clear how to perform this operation, and in the absence of the indentification of a distinct inorganic compound (*e.g.* calcium sulphate in a composite material with PLA for example) the whole sample will be analysed without any correction factor introduced for inorganic content.

As previously discussed, sodium has a single stable isotope and does not have a role in stable isotope analysis. There is no nitrogen in the sample, and so carbon, hydrogen, oxygen, and sulphur stable isotopes will be used to assist in the assignment of total bio-based

content. Given the significant overlap in the stable isotope ratios of these elements when comparing bio-based and fossil sourced materials, only a combination of analyses might be able indicate a possible renewable origin. Ideally a bio-based product would be downstream of a C_4 plant feedstock, which would give the most clarity in the stable isotope analysis. Any certification deriving from stable isotope analysis would need to be weary of inadvertly discouraging the use of wheat waste (and other plants with C_3 plant metabolism) and creating a bias towards sugarcane and corn (C_4 plants) just because they will be easier to certify without necessarily considering the broader environmental sustainability issues of bio-based products. Alternatively a radiocarbon analysis in combination with a declaration from the supplier describing the chemical production process would resolve the percentage total bio-based content. Ideally chemical suppliers will disclose the ingredients of their formulations but it is not to be expected. In the absence of chemical disclosure it would be beneficial if the supplier could guide sample preparation procedures. For example, if volatiles are present in a chemical product of low water content, then drying methods would best be avoided in case of unintended fractionation.

The use of molecular composition to assign a total bio-based content is not applicable to sodium laureth sulphate because of the two rival production methods, one based on ethylene polymerisation and the other based on coconut oil processing are very much downstream of the original feedstock chemicals (**Figure 4.2**). It is impossible to resolve from chromatography coupled with spectroscopy the source of the sodium laureth sulphate, although deducing the structure of the molecule if otherwise unknown helps refine the number of possibilities concerning its origin.

6 List of standards

ASTM D297	Standard test methods for rubber-products chemical analysis
ASTM D1762	Standard test methods for chemical analysis of wood charcoal
ASTM D2650	Standard Test Method for Chemical Composition of Gases By Mass Spectrometry
ASTM D5017	Standard Test Method for Determination of Linear Low Density Polyethylene (LLDPE) Composition by Carbon-13 Nuclear Magnetic Resonance
ASTM D5291	Standard test methods for instrumental determination of carbon, hydrogen, and nitrogen in petroleum products and lubricants
ASTM D6866	Standard test methods for determining the biobased content of solid, liquid, and gaseous samples using radiocarbon analysis
ASTM D7026	Standard guide for sampling and reporting of results for determination of biobased content of materials via carbon isotope analysis
ASTM D7399	Standard Test Method for Determination of the Amount of Polypropylene (PP) in Polypropylene/LDPE Mixtures Using Infrared Spectrophotometer (FTIR)
ASTM D7455	Standard practice and sample preparation of petroleum and lubricant products for elemental analysis
ASTM D7459	Collection of integrated samples for the speciation of biomass (biogenic) and fossil-derived carbon dioxide emitted from stationary emission sources
ASTM D7806	Standard Test Method for Determination of the Fatty Acid Methyl Ester (FAME) Content of a Blend of Biodiesel and Petroleum-Based Diesel Fuel Oil Using Mid-Infrared Spectroscopy
ASTM E1757	Standard practice for preparation of biomass for compositional analysis
CEN/TS 16137	Plastics - Determination of bio-based carbon content

EN 1787	Foodstuffs - Detection of irradiated food containing cellulose by ESR spectroscopy
EN 12630	Fruit and vegetable juices - Determination of glucose, fructose, sorbitol and sucrose contents - Method using high performance liquid chromatography
EN 14774-3	Solid biofuels - Determination of moisture content - Oven dry method - Part 3: Moisture in general analysis sample
EN 14780	Solid biofuels – sample preparation
EN 15104	Solid biofuels - Determination of total content of carbon, hydrogen and nitrogen – Instrumental methods
EN 15413	Solid recovered fuels. Methods for the preparation of the test sample from the laboratory sample
EN 15440	Solid recovered fuels – Method for the determination of biomass content
EN 15443	Solid recovered fuels – Methods for the preparation of the laboratory samples.
EN 15289	Solid biofuels — Determination of total content of sulphur and chlorine
ENV 12140	Method for determination of stable carbon isotope ratio ($^{13}C/^{12}C$) of sugars from fruit juices, using isotope ratio mass spectrometry
ENV 12142	Method for determination of stable hydrogen isotope ratio (2H/1H) of water from fruit juices, using isotope ratio mass spectrometry
ENV 13070	Fruit and vegetable juices - Determination of the stable carbon isotope ratio ($^{13}C/^{12}C$) in the pulp of fruit juices - Method using isotope ratio mass spectrometry
ISO 356	Essential oils – Preparation of test samples
ISO 1795	Rubber, raw natural and raw synthetic – Sampling and further preparative procedures
ISO 5089	Textiles - Preparation of laboratory test samples and test specimens for chemical testing

ISO 8432	Essential oils - Analysis by high performance liquid chromatography - General method
ISO 10210	Plastics – Methods for the preparation of samples for biodegradation testing of plastic materials
ISO 11543	Modified starch - Determination of hydroxypropyl content – Method using proton nuclear magnetic resonance (NMR) spectrometry
ISO 15512	Plastics - Determination of water content
ISO 22972	Essential oils – Analysis by gas chromatography on chiral capillary columns – General method
TAPPI T257	Preparation of wood for chemical analysis

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