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The Power of Stable Isotope Dilution Assays in Brewing

The quantification of trace substances in raw materials, mash, wort and beer is becoming more frequently required by brewers. Stable isotope dilution assay (SIDA) coupled to gas chromatography mass spectrometry or liquid chromatography mass spectrometry instrumentation is a sensitive and highly specific technique for precise quantification of trace substances from complex matrices. The current paper elucidates the basic principles of stable isotope dilution assays and presents practical applications related to brewing. It emphasizes the importance of isotope standards in multi-step sample clean-up procedures and answers the question why SIDA is (so far) not widely used in brewery quality control.

Descriptors: mass spectrometry, stable isotope dilution analysis, SIDA, beer staling, contamination

1 Introduction - Isotopes, isotopologues and their relevance for brewing science

The denotation isotope traces back to Nobel Prize winning chemist Frederick *Soddy*, who discovered their existence almost 100 years ago [1]. Isotopes are atoms of a particular chemical element featuring equal numbers of protons but differing numbers of neutrons. Due to the varying numbers of neutrons the atomic mass of isotopes differs. Many chemical elements have a natural distribution of stable isotopes and radioactive isotopes (radioisotopes). In contrast to radioisotopes stable isotopes do not decay by emission of alpha, beta or gamma radiation. Carbon (C) for example, consists of stable ^{12}C and ^{13}C isotopes, as well as of radioactive ^{14}C. Hydrogen (H) exists as stable ^{1}H (protium), $^{2}H/D$ (deuterium) and beta-decaying $^{3}H/T$ (tritium). Table 1 illustrates the main elements of biomass. They appear in a varying mixture of several stable isotopes. In consequence of their mass and relative abundance, isotopes influence the molecular mass of organic molecules. Several terms to describe the mass of organic molecules are used, their distinction is of greatest importance in stable isotope analysis. Table 2 shows the nominal mass and the monoisotopic mass of ethanol including their calculation. While the nominal mass is calculated using the integer mass of the most abundant elements of the molecule, the monoisotopic mass is calculated from the exact masses. Table 2 also illustrates the exact molecule mass of four randomly selected ethanol isotopologues. Isotopologues are molecules that vary only in the isotopic nature of their elements. Dependent on type and number of stable isotopes contained in a molecule different exact molecular masses result. Differentiation of isotopologues is therefore possible by mass spectrometry. The relative abundance of each isotopologue in a sample is called the isotopic pattern. Besides their natural occurrence specific isotopologues can be chemically synthesized. This enables two ways of analytical approaches:

Analysis of the natural isotopic pattern of organic substances

Physical processes such as the evaporation of water and biochemical reactions such as photosynthesis lead to very low but measurable shifts in the relative abundance of isotopes. Material of e.g. plants and animals obtain a specific isotopic pattern that allows their assignment to a regional area or a manufacturing process. The fact that stable isotope abundances vary enables stable isotope analysis. Techniques such as isotope ratio mass spectrometry (IR-MS) and site-specific natural isotope fractionation nuclear magnet resonance spectroscopy (SNIF-NMR) are powerful tools to evaluate food origin and judge food authenticity [2, 3, 4, 5].

Dilution of the natural isotopic pattern by chemically synthesized isotopologues

Chemical synthesis enables the preparation of organic molecules enriched in naturally low abundant and "heavy" isotopes such as e.g. ^{13}C, D, ^{18}O and ^{15}N. These "heavy" isotopologues are successfully used as tracers in in-vivo studies for biomedical research, biochemistry or metabolomics [6, 7, 8]. In food chemistry and related scientific fields stable isotope standards are frequently used in stable isotope dilution assays (SIDA). SIDA is an internal standard assay in which analyte and standard are isotopologues. SIDA was first published by *Sweely* et al. in 1966 [9]. Today it is widely accepted for precise (trace) substance quantification from complex matrices [e.g. 10, 11, 12] and more and more frequently required by brewers.

The current paper elucidates the basic principles of stable isotope dilution assays. It emphasizes the importance of isotope standards in trace analysis and multi-step sample preparation procedures. Prerequisites for SIDA and specifications for stable isotope standards are summarized. To underline the power of SIDA practical applications related to brewing are presented.

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Tables and figures see Appendix

2 Basic principles of stable isotope dilution assays

Natural organic compounds contain certain amounts of low abundant heavy stable isotopes (cf. Table 2). If those are spiked with chemically synthesized standards enriched in "heavy" D, ^{13}C or ^{18}O isotopes, the natural isotopologues are diluted. This is the origin of the term dilution in SIDA (cf. Fig. 1). SIDA is a special case of an internal standard assay in which the only difference between analyte and standard is a small shift in molecular mass. The idea of internal standard assays, using a standard as similar as possible to the analyte, is perfectly accomplished. In SIDA the ratio of analyte and standard is an isotope ratio measured by mass spectrometry (MS).

2.1 How to perform a SIDA?

Firstly SIDA requires the identification of the analyte (target) and the examination of its mass spectrum. Depending on the ionization mode, molecular ions and fragment ions can occur. In the mass spectrum characteristic and prominent peaks have to be identified. Peaks in a mass spectrum represent the mass-to-charge ratio (m/z) of fragment ions, the peak intensity indicates the relative abundance of each ion. In MS language the most prominent peak is called base peak. Its intensity is set to a relative abundance of 100 %. The intensities of other fragment ions are related to the base peak. When the analyte mass spectrum is recorded an isotopologue of the analyte, a so called stable isotope standard, has to be synthesized or purchased. Its mass spectrum has to be recorded as well. Compared to the analyte the isotope standard needs to exhibit corresponding characteristic ions with shifted m/z ratios. The difference in m/z depends on the type and number of stable isotope labels in the standard molecule, or in the fragment ion triggering the peak. The characteristic ions are used to identify and quantify analyte and standard. They are the basis for quantification via SIDA. Assuming that a proper stable isotope standard is chosen a SIDA may be performed. Figure 2 illustrates the principle steps of a SIDA equaling a conventional internal standard assay. At the very beginning of sample preparation the sample is spiked with (isotope) standard. Equilibration is reached by stirring, mixing or shaking. Dependent on complexity of sample matrix and chromatographic setup, sample preparation and target isolation is accomplished. In SIDA the nearly identical chemical structure of analyte and stable isotope standard results in almost identical physical and chemical properties. Boiling point, solubility, acidity, and reactivity are highly similar. Also characteristics of isotopologues concerning extraction, chromatography and ionization match (cf. Fig. 2). Sample preparation removes matrix components and coincidental usually enriches analyte and standard. The resulting extract is injected onto a chromatography system. Ideally chromatographic setup is capable of base line separation of remaining matrix compounds from analyte and standard isotopologues. Analyte and stable isotope have identical or almost similar retention times. Due to co-elution MS is needed for differentiation. To distinguish analyte and standard and to increase sensitivity SIDA is commonly coupled to selected ion monitoring-mass spectrometry (SIM-MS). SIM-MS is a technique in which only a small number of selected ions is monitored. In SIDA the selected ions correspond to the characteristic fragment ions of analyte and standard (cf. Fig. 3). The ratio of analyte and standard is an isotope ratio. From a known amount of isotope standard, analyte concentration can be calculated using SIM-MS peak areas. Figure 3 illustrates an example for the relation of fragment ions and SIM-MS. It shows the mass spectra of linalool and D_5-linalool, as well as a SIM-MS chromatogram of their mixture. In this example two ions (m/z = 93 and m/z = 98) are used for quantification. They are called analyte and standard quantifier ions. Two more ions (m/z = 121 and m/z = 126) are used to detect matrix effects and assure compound identity. These ions are called qualifier ions. The intensity of quantifier and qualifier ions shall have a constant ratio that is given by the fragmentation pattern of target and standard. If those ratios do not match, matrix components may co-elute and interfere with quantification. When tandem-MS instrumentation (MS/MS) is applied the rather simple SIM-MS technique can be extended. MS/MS enables selected reaction monitoring (SRM) experiments, formerly named multiple reaction monitoring (MRM). SIM-MS and SRM are very similar. As the name implies instead of selected ions selected fragmentation reactions are monitored. In SRM the first mass analyzer selects precursor ions. Fragmentation of precursor ions produces specific product ions that are monitored. Precursor and product ions were formerly called parent and daughter ions. SRM experiments add further selectivity and specificity to MS detection.

2.2 Prerequisites for SIDA and specifications of stable isotope standards

To perform a SIDA a mass spectrometer coupled to liquid chromatography (LC-MS) or gas chromatography (GC-MS) as well as suitable stable isotope standards (isotopologues of the target compounds) are required. While prices for simple GC-MS and LC-MS systems are constantly decreasing, costs for commercial stable isotope standards or their chemical synthesis are undoubtedly the disadvantages of SIDA. Today only a limited selection of labeled substances is available from commercial suppliers. Prices for labeled standards exceed those of unlabeled substances by a multiple: Analytical grade acetaldehyde (C_2H_4O) costs less than 10 €/gram, D_3-acetaldhyde costs about 200 €/gram and the $^{13}C_2$ isotopologue more than 600 €/gram. Many labeled substances are simply not available. Either they have to be purchased from upscale special suppliers or have to be synthesized. Indeed prices for labeled standards or precursors seem to be high on first view, but have to be judged with care. SIDA is mainly applied in trace analysis and therefore only a few ng or µg standard are needed per sample. In case one gram of a labeled educt costs 1000 € and the yield of a synthesis is only 10 %, 100 mg isotope standard cost 1000 €. In trace analysis, e.g. requiring 10 µg standard per sample, almost 10,000 runs may be performed with 100 mg standard. In this example costs for isotope standards add up to 0.10 € per sample [13]. Compared to costs for solvents and manpower this investment appears to be insignificant. In food chemistry mainly D, ^{13}C, ^{18}O and ^{15}N isotopes are used to label molecules. The choice favoring one or the other label depends on the chemical structure of the analyte, the availability and price of labeled precursor molecules or reagents, and on the route of chemical synthesis. An important aspect of standard synthesis is the robustness of the label. Carbon-carbon or nitrogen-carbon bonds are usually very stable, thus ^{13}C and ^{15}N labels are very robust. ^{18}O or D labels are susceptible to exchange reactions if

they are activated by neighborhood of aromatic rings or carbonyl functions. Before ^{18}O or D labeled standards are used in SIDA label robustness has to be proven. Labeling with D (deuteration) is very common. D-enriched chemicals are comparably cheap and D is usually much easier to introduce than ^{13}C labels. Figure 4 illustrates some popular examples of deuteration reactions. Reagents and parameters used in deuteration reactions are usually mild. As mentioned above the advantage of SIDA is the matching properties of analyte and standard. However the isotope effect (IE) has to be taken into consideration. IE describes marginal differences in chemical and physical properties of isotopologues due to very low energy differences caused by differing atomic masses. IE is of special importance when D labels are used. The mass difference between ^{1}H and D (D has twice the mass of ^{1}H) is proportionally much higher than those between ^{12}C and ^{13}C for example (cf. Table 1). IE influences reactivity and boiling point of isotopologues. It can cause very weak but measurable chromatographic separation (Fig. 3) and biochemical reactivity. To minimize IE only the necessary number of labels shall be introduced and uniformly labeled standards are not desired. The number of labels is also very important for quantification by mass spectrometry. An accurate quantification requires a definite distinction of analyte and isotope standard. Signals of either the molecular ions, the base peaks or of other characteristic ions need to carry stable isotope labels. When analyte and standard mass spectra differ, ions with slightly shifted m/z are perceptible and SIM-MS enables differentiation and quantification. In some cases isotopologue differentiation is difficult due to the natural abundance of heavy isotopes. Organic material contains roughly 1 % of ^{13}C (cf. Table 1). Therefore, molecules and their fragments likely include ^{13}C. They do not only exhibit their monoisotopic mass but also monoisotopic masses +1 or +2 or more. To avoid a signal overlap the number of labels and the resulting mass shift has to be sufficient. The minimal number of labels required strongly depends on size of qualifier ion monitored in SIM-MS. The higher its mass the more labels are needed to avoid signal overlap. In practice usually labels are introduced to exhibit m/z +3 of the prominent ions.

2.3 Calibration procedures

The quantification via SIDA is based on integration of quantifier m/z traces of analyte and isotope standard. In addition, a calibration is needed to establish a correlation between isotopologue ratio and signal intensity. In SIDA calibration is realized by a series of experiments in which the intensities of isotope standard and analyte mixtures of different concentrations are measured by GC-MS or LC-MS. Whereas standard concentration remains constant, analyte concentration is varied [14]. Good stable isotope standards contain non or only minimal amounts of unlabeled material. Appropriate labeling avoids spectral overlap. In those cases calibration curves usually linear for molar analyte to standard ratios within at least a factor of 25. In case stable isotope standards are labmade nuclear magnetic resonance spectroscopy (NMR) experiments are needed to prove standard purity. Otherwise systematic errors may lead to imprecise quantification. In addition the chemical and isotopic purity of isotope standard solutions for routine analyses can, and should be, frequently checked by 1:1 mixing with pure analyte solution. By this, correction factors can be calculated prior to analysis.

3 The power of SIDA in brewing

Hops, mash, wort and especially beer are particularly challenging analytical matrices. The presence of numerous compounds strongly differing in molecular size, physical, and chemical properties hinders precise standard analysis of trace components. Nevertheless brewers more frequently demand detailed information on trace level (ng/kg or µg/kg) substances in raw materials, wort and beer. Today chromatographic systems are increasingly coupled to mass spectrometry (MS). If employed properly, MS is a very powerful tool. In comparison to basic GC and LC detectors MS enables substance characterization and identification. MS enables non-target screenings and SIM-MS or SRM-MS/MS modes which clearly increase the selectivity and sensitivity of detection. SIDA reduces matrix effects and precise substance quantification is enhanced. Trace analysis in brewing primarily concentrates on flavor active molecules and their precursors. Also rising requirements of food safety call for frequent analysis of food contaminants such as mycotoxins [15], phthalates, bisphenol A (BPA), and its reaction products [16, 17, 18]. The state of the art instrumentation provides highly sensitive analysis, however trace analysis in complex beer matrix is still challenging. In precise trace substance quantification frequently multi-step sample clean-up procedures are needed, hence the choice of an adequate standard has to be carried out carefully.

3.1 Disadvantages of conventional external and internal standards in trace analysis

Sugars, proteins, polyphenols, alcohols or organic acids mask trace targets during beer analysis. Matrix effects frequently interfere with or even preclude substance identification and quantification. In addition to modern GC-MS and LC-MS instrumentation analytical setups have to include appropriate techniques of sample clean-up, target isolation, calibration, and sometimes derivatization. To extract and enrich target substances countless approaches of sample clean-up are known. To extract volatiles prior to GC analysis liquid-liquid extraction procedures coupled to solvent assisted flavor evaporation (SAFE) [19] or simultaneous distillation extraction methods (SDE) [20] are common. In order of miniaturization and automation several static or dynamic headspace methods are available. Target enrichment is reached by solid phase micro extraction (SPME) [21, 22, 23], stir bar sorptive extraction methods [25] or purge and trap techniques [25]. To take advantage of LC-MS sensitivity, flexibility and accuracy matrix load is usually decreased prior to LC separation. Here mainly target specific on- and offline-solid-phase extraction (SPE) procedures are used [26, 27]. However, the more intense sample clean-up or derivatization procedures, the more sources of analyte losses and errors have to be considered in quantification and calibration. In case external calibration is performed parameters of sample preparation and analysis have to be maintained perfectly constant. Calibration and quantification are only correct if e.g. temperature, extraction time, sample or headspace volume is perfectly steady. Decreasing adsorptive capacity has to be taken into account when SPME fibers or Tenax® resins are used. In those cases numerous repeat calibrations and saturation experiments are needed. However it is still debatable if quantification is right [28]. When it comes to trace analysis in biological matrices (such as hop products, malt, beer and wort) fluctuations from sample to sample will quickly

be recognizeable. Unsteady and changing sample constitution strongly influences target isolation. The disadvantages of external standard quantifications are obvious: Matrix effects and analyte loss during sample preparation are not included. Also the analytical setup has to be highly reproducible. Many problems can be reduced by internal calibration, however accuracy is very much dependent on the structure of the internal standard. Structural differences between internal standard and analyte can cause severe differences in chemical and physical properties. Equilibration of standard and matrix may not be reached and enrichment may be disproportionate. As targets are frequently labile, reactive or volatile during analysis standard recovery differs from analyte recovery. In order to reduce differences correction (response) factors are usually determined in water or water/ethanol mixtures. Empirically results transferred from aqueous model solutions do not properly reflect reality. In all these cases quantification might be incorrect or at least imprecise. Very volatile or non-volatile analytes may also require derivatization prior to GC-MS and LC-MS. Derivatization is a crucial and often deficient step in sample preparation. Analyte and standard need to react likely identically with derivatization agents over an acceptable concentration range. In case that structurally different internal standards are used systematic errors cannot be excluded. If analyte concentration is high (mg/kg range) and reliable references are available, cost effective standard GC and LC methods are applicable. The lower the target concentration, the higher are the demands for accuracy. Therefore more sensitive GC-MS and LC-MS instrumentation is used, which results in higher necessity of stable isotope standards. Improper standards can cause unproportional discrimination in the GC injection port or during chromatography. Above all the ionization in MS is critical. Some ions may be suppressed or enhanced, thus external response factors may not be valid. In GC-MS electron impact ionization (EI) MS leads to fragmentations of the target molecules and quantification must be performed by SIM or total ion current (TIC). Equal amounts of unlabeled internal standard and analyte will usually not give similar SIM or TIC response. To our experience correction factors are valid only in a low analytical range. Therefore, quantification of homologous compounds occurs reliable by internal standard analysis, e.g. quantification of stearic acid by heptadecanoic acid. Thus, the use of heptadecanoic acid to accurately quantify i.e. unsaturated alpha-linolenic acid is questionable.

3.2 SIDA in LC-MS and GC-MS applications

Challenges going along with trace analysis indicate SIDA coupled to MS as the method of choice. Approximate physical and chemical properties of analyte and isotope standard assure equal behavior during extraction, concentration and derivatization. Also chromatographic and MS properties match. Loss of standard during sample preparation exactly equals the loss of analyte and vice versa. The lower the target concentration and the higher its volatility and/or reactivity the more essential is the use of isotopologues/isotope standards. If spiking with standard is carried out precisely, SIDA excludes systematic errors.

In GC-MS applications all systematic errors arising from external and internal calibration vanish when using SIDA. Ionization techniques used in GC-MS are electron impact (EI) and chemical ionization (CI). CI can be operated in positive (PCI) and negative (NCI) mode. Especially NCI fits sensitively for electro negative compounds like carbonyls and subsequently eliminates many matrix effects. MS setups use molecule fragments for identification and quantification. The fragmentation intensities of analyte and isotope standard are highly similar and, with respect to the calibration, quantification can be done by direct comparison of the respective m/z. For all these benefits SIDA is also used in more sophisticated LC-MS, however this is slightly more challenging. In LC-MS substances of diverse polarities and molecular masses can be analyzed. Their ionization is reached by electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). Whereas in GC-MS the peak area of the isotope standard remains constant if the analyte co-elutes, competition issues in ESI and APCI can be observed. In APCI ionization enhancement was reported [29, 30]. Ionization enhancement means that higher analyte intensities and peak areas result if stable isotope standard co-elutes. Unlike in LC-ESI-MS internal standard peak areas sometimes decrease with increasing concentration of co-eluting analyte and vice versa. This effect is called internal standard signal suppression. It occurs if internal standard or analyte concentration is high, or if one or the other is present in high excess [31]. Indeed potential competition issues could be interpreted as a sign of irreproducible response, still co-eluting internal standards are the safest way to eliminate matrix effects and minimize systematic during sample preparation. To avoid inaccurate quantification via LC-MS careful calibration is needed. To examine potential competition issues analyte and standard peak areas should be recorded in single runs as well as in mixtures of varying concentrations. During analysis standard concentration should be adjusted to virtually match analyte concentration.

3.3 Applications of SIDA in brewing

Trace analysis in brewing mainly concentrates on flavour active substances such as carbonyls, sulfur compounds, hop oils etc. Also detection of trace contaminants derived from raw materials or packaging materials gains importance.

Flavor active products of Strecker-type reactions, fatty acid degradation or fermentation have extraordinary low flavor and odor thresholds and infinitesimal concentration changes may strongly affect beer flavour. To identify changes within processing and storage precisely reliable methods for quantification are needed. High volatility and reactivity of flavor active molecules are continuous analytical challenges, thus use of deuterated and ^{13}C stable isotope standards for quantification is very reasonable. SIDAs have been developed for a huge number of carbonyls, in most cases GC-MS setups are used. *Fritsch* and *Schieberle* [32] quantified 21 odorants from Bavarian pilsener type beers using SAFE distillation technique and stable isotope labeled standards such as $^{13}C_2$-acetaldehyde, D_6-dimethyl sulfide, D_2-methylbutanal and D_2-phenylethanol. Related publications include the quantification of β-damascenone [33], furaneol [34], *trans*-2-nonenal [35] or aromatic alcohols [36]. The stable isotope labeled standards were predominantly labmade, therefore routes for their chemical synthesis are mainly included in the belonging publications. In recent years many solvent and time consuming multi-step sample preparation procedures were displaced by headspace-SPME techniques. As described before SPME is strongly dependent on factors such as salt addition and

sampling time. Also fibre capacity decreases from run to run and SPME is sensitive to changes in sample composition. To minimize procedural errors SPME should be necessarily coupled to SIDA [21, 37]. A SPME method using labmade D_2-linalool as internal standard to quantify (*R*)- and (*S*)-linalool was published by *Steinhaus* et al. [21]. Correspondingly *Scherb* et al. [37] verified D_6-dimethyl sulfide as a very reliable internal standard for quantification of dimethylsulfide via SPME and high resolution GC-MS. Both working groups concluded that internal isotope standards resulted in a good run-to-run reproducibility, low standard deviation and precise quantification. To improve their chromatographic properties and enable highly selective and sensitive detection by GC-NCI-MS alkanals, alkenals and alkadienals are frequently converted to their corresponding fluoro oxime derivates by reaction with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (PFBHA). In some cases PFBHA derivatization is performed on the SPME fibre [22]. Here stable isotope standards, can be used to assure precise quantification even if an additional derivatization step is included. An appropriate study has been presented by *Schmarr* et al. [38], though exclusively uniform labeled D_{16}-octanal was used as internal isotope standard. The contamination of food by harmful, toxic or even carcinogenic substances is a public health concern. In comparison to other foodstuff beer production and packaging results in a pure and rarely contaminated product. To prove product purity and to detect trace contaminations highly sensitive analytical assays are needed. A frequent monitoring for mycotoxins, biogene amines or nitrosamines is even proposed to be included in HACCP protocols [39]. Reliable SIDAs have been published for the determination of N-nitrosodimethylamine (NDMA) using D_6-NDMA [40], acrylamide e.g. by $^{13}C_3$-acrylamide [41] standard or D_3-acrylamide [42], *ortho*-phenylphenol by a D_2 to D_{10}-ortho-phenylphenol stable isotope mixture [43], as well as Di- and trihydroxybenzenes using corresponding D_3-analogues [44]. SIDAs for zerealenone and ochratoxin A in beer have been published by *Zöllner* et al. [45] and *Lindenmeier* et al. [46]. In general mycotoxin analysis is a complicated and multifaceted discipline. The application of SIDA in mycotoxin analysis has been comprehensively reviewed by *Rychlik* and *Asam* [13].

Apart from raw materials the migration of packaging material can cause food contaminations. The migration of food contact materials into beverages has received a lot of attention in the past. Food and beverage cans posses an inner coating to protect the foodstuff from metal contact and to prevent metal corrosion due to low product pH. Beer can coatings are mainly based on bisphenol A (BPA) reaction products, such as bisphenol A diglycidyl ether (BADGE) and higher oligomers. The analysis of BPA and BADGE is usually carried out by GC-MS and LC-MS setups. D_{16}-BPA is commercially available, protocols for the chemicals synthesis of e.g. D_4-BPA as well as D_{14}-BAGDE are published [17, 18]. For BPA and BADGE are ubiquitous in nature, the precise quantification requires the application of internal stable isotope standards [47].

4 The identification of (bio)chemical pathways by isotope labeling

Stable isotope standards offer far more options than "only" quantification via SIDA. Labeled substrates can be used to trace oxidative or enzymatic formation and degradation pathways of e.g. aroma compounds.

An example for a yeast catalyzed biosynthesis was presented by *Garbe* [48]. In this work the formation of aroma active gamma-nonalactone isomers was studied using tetra deuterated, single deuterated and ^{18}O-labeled 13- and 9-hydroxyoctadecadienoic acids. Stable isotope labeled fatty acids have also been successfully used in elucidation of lipoxygenase (LOX) pathways which are of greatest importance for fatty acid degradation during malt processing and wort preparation [49]. Maillard reaction and especially Strecker-type reactions are an efficient source of aroma active aldehydes formed during wort boiling and beer storage. For their enormous importance in food processing and food chemistry numerous investigations using stable isotope labeled substances have been published. For the complexity of those reactions mostly model systems have been applied. A detailed and comprehensive discussion would lead to far, important examples for stable isotope labeling experiments are amongst others given by *Blank* et al. [50] as well as *Hofmann* and Schieberle [51, 52].

The major advantage of labeled substrates is that reaction products can be traced with help of D-, ^{13}C-, ^{18}O- or ^{15}N-labels. Declarations on reaction mechanisms can be predicted, assuming labels are stable and experimental setup is adequate. As described above, D and ^{18}O are susceptible to exchange in labile positions e.g. next to carbonyl functions. If this fact is disregarded misinterpretations are likely. An example for this was presented by *Noel* et al. [53]. In reference to *Owades* and *Jakovac* [54] ^{18}O oxygen was injected into bottle headspace and oxidative reactions were monitored. Both research groups found considerable oxidative damage, e.g. ^{18}O was traced in oxidized polyphenols and isohumulones. In addition to the experiment of Owades and Jakovac, Noel et al. tried to trace *trans*-2-nonenal and its degradation products (nonenoic acid, 3-hydroxynonanal). They assumed the presence of ^{18}O-labels in those substances would evidence oxidative fatty acid degradation in bottled beer. From the fact ^{18}O-labelled reaction products could not be traced they concluded no fatty acid oxidation occurs. This conclusion in not sufficient, as figure 5 shows the ^{18}O label of *trans*-2-nonenal is exchanged by water addition and elimination [Garbe 2011, unpublished results]. The intermediate occurrence of ^{18}O-labelled *trans*-2-nonenal will exchange with $H_2{}^{16}O$ (water), which always results in ^{16}O labeled *trans*-2 nonenal independent from formation pathways. Therefore Noel et al. interpretations and conclusion might be wrong. Nevertheless proper ^{18}O labeling is very interesting. Stable incorporated ^{18}O oxygen can prove oxidative damage and indicate (bio)chemical reactions. An interesting example of ^{18}O-labeling is covered in an up to date series of publications of *Intelmann* et al. [55, 56, 57]. Their works were focused on changes in the sensory perception of bitter compounds during beer storage. By ^{18}O-labeling experiments the site of stable incorporated ^{18}O oxygen was determined, thus degradation mechanisms could be evidenced. For the first time a conclusive mechanism of *trans*-isohumulone transformation into tri-and tetracyclic compounds was presented.

The major advantages of stable isotope labeling should be obvious: It is the superior quality of observations and declarations that can be derived from those experiments. Results of conventional

experiments are usually based on unspecific correlations e.g. between increasing product and decreasing educt concentrations. Stable isotope labeling provides detailed information on reaction mechanisms, educt and product concentrations, conversions rate and is liable to prove reaction mechanisms.

5 Summary and perspectives

Stable isotope dilution analysis is a very powerful technique in brewing science, food chemistry and food safety. In brewing science and quality control stable isotope labeled substances offer two highly relevant and versatile applications:

Stable isotope labeled molecules can be used as (bio)chemical tracers

The transformation and degradation of labeled educts help to gain detailed information on (bio)chemical reactions such as fatty acid degradation, Strecker-type reactions or specific enzymatic conversions. In contrast to results of conventional experiments which are usually based on unspecific correlations (e.g. between increasing product and decreasing educt concentrations), stable isotope labeling provides detailed information and is liable to prove reaction mechanisms.

Isotope standards are successfully used in stable isotope dilution assays (SIDA)

SIDA used with LC-MS or GC-MS instrumentation enables precise trace substance quantification from complex matrices. Problems that arise by conventional internal or external calibration are avoided, highly sensitive SIM- respectively SRM-MS modes boost the specificity and accuracy of beer analysis. SIDA is mainly used in the analysis of volatile and reactive molecules such as staling aldehydes, dimethyl sulfide or hop oils. Also SIDA is the method of choice when quantification of trace contaminants such as phthalates, bisphenol A, BAGDE, NDMA or fungal mycotoxins is needed. The advantages of SIDA originate from the nearly identical chemical structure of analyte and standard. The internal stable isotope standard is an isotopologue of the analyte, thus they are chemically and physically as near identical as possible. Characteristics during sample cleanup, chromatographic separation and ionization are practically identical. SIDA excludes systematic errors and provides superior analytical specificity and sensitivity.

Despite its benefits stable isotope analysis has hardly entered brewery quality assurance labs and is still not state of the art in brewing research. The major disadvantage is doubtless the necessity for costly stable isotope standards and MS techniques. Even commercial suppliers constantly increase their offerings, only a limited number of brewing relevant stable isotope standards is commercially available today. Dependent on the type of label (^{13}C, ^{15}N, D or ^{18}O) and on their chemical structure prices for labeled standards exceed those of unlabeled substances by a multiple. Isotope standards can easily cost the hundredfold than corresponding unlabeled analytical grade chemicals. Prices indicate that the chemical synthesis is frequently complex and time consuming, so far low demand for isotope standards prevents a serious price competition between suppliers. In addition to an impeded access to isotope standards MS techniques seem to challenge brewers: The latest equipment represents a considerable investment and skilled staff is required to exploit the capabilities of GC-MS and especially LC-MS equipment. In the near future MS instrumentation will surely become more popular in brewing related labs and research institutes. Rising demands regarding complex analytical challenges such as food safety issues will definitely require more frequent use of SIDA.

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Appendix

Element	stable isotopes	exact atomic mass [Dalton]	relative abundance [%]
Hydrogen	^{1}H	1.007825037	99.9855
	^{2}H	2.014101787	0.0145
Carbon	^{12}C	12.00000 (scale basis)	98.892
	^{13}C	13.003354	1.108
Nitrogen	^{14}N	14.003074	99.6337
	^{15}N	15.00011	0.3663
Oxygen	^{16}O	15.99491464	99.7587
	^{17}O	16.9991306	0.0374
	^{18}O	17.99915939	0.2039
Sulfur	^{32}S	31.972074	95.0018
	^{33}S	32.9707	0.750
	^{34}S	33.96938	4.215
	^{35}S	35.96676	0.02

Table 1 **Bioelements exist as a mixture of stable isotopes. Isotopes differ in their relative abundance, due to the varying number of neutrons their exact atomic mass differs as well**

Table 2 **Several terms to describe the mass of molecules exist. The table illustrates that the nominal and the monoisotopic masses of ethanol differ. With help of four selected isotopologues differences in exact molecular masses are illustrated**

Ethanol (C_2H_5OH)				
monoisotopic mass	(2*12+6*1.00783+1*15.995) = 46.042			
nominal mass	(2*12+6*1+1*16) = 46			
selected isotopologues	$H_3{}^{12}C$–^{16}OH	$H_3{}^{12}C$–^{18}OH	$H_3{}^{13}C$–^{16}OH	$H_3{}^{12}C$–^{16}OD
elemental formula	$^{12}C_2{}^1H_5{}^{16}O^1H$	$^{12}C_2{}^1H_5{}^{18}O^1H$	$^{12}C^{13}C^1H_5{}^{16}O^1H$	$^{12}C_2{}^1H_5{}^{16}OD$
exact mass	46.042	48.046	47.048	47.045

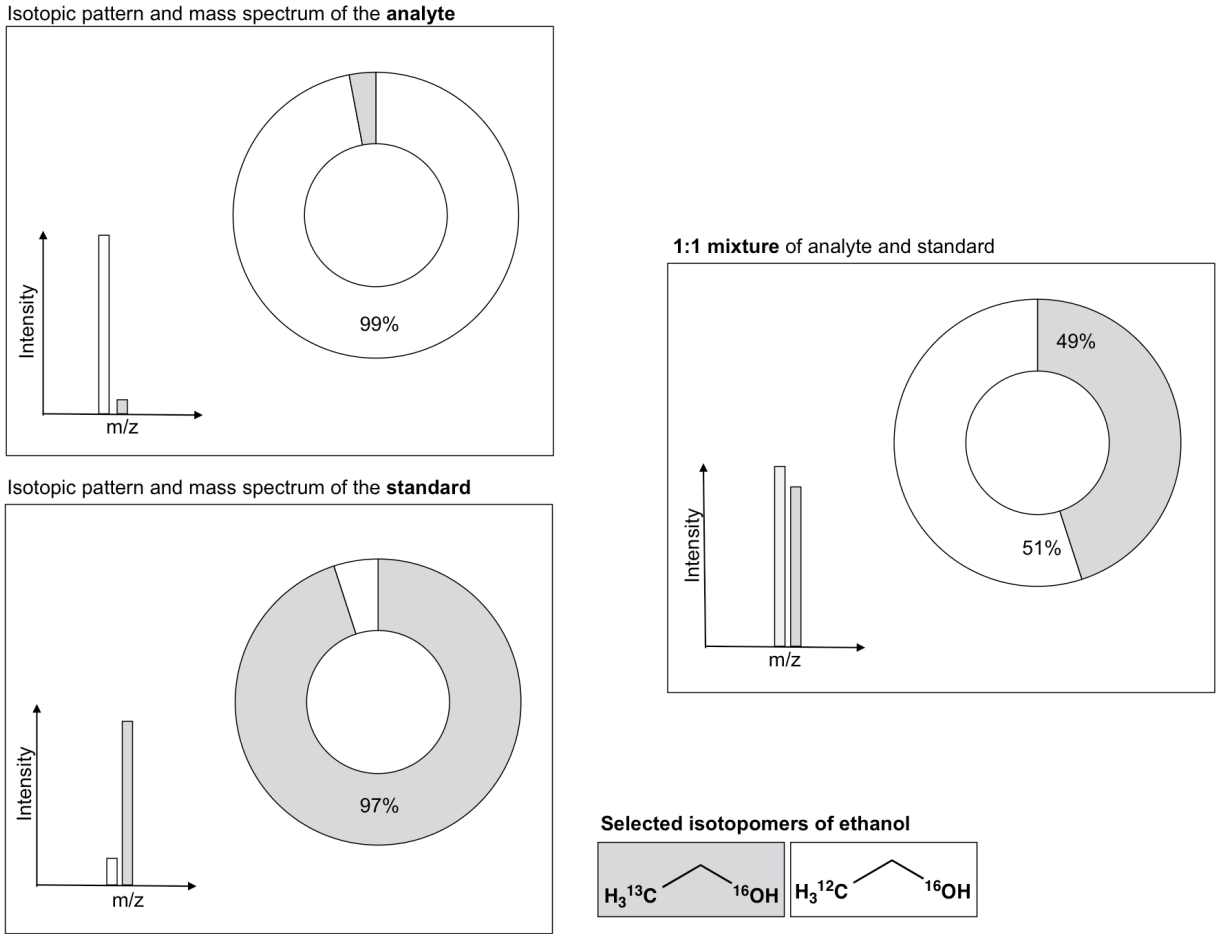

Fig. 1 **Natural analyte and chemically synthesized stable isotope differ in their isotopic pattern and mass spectrum. Mixing results in a dilution of the original isotopic pattern**

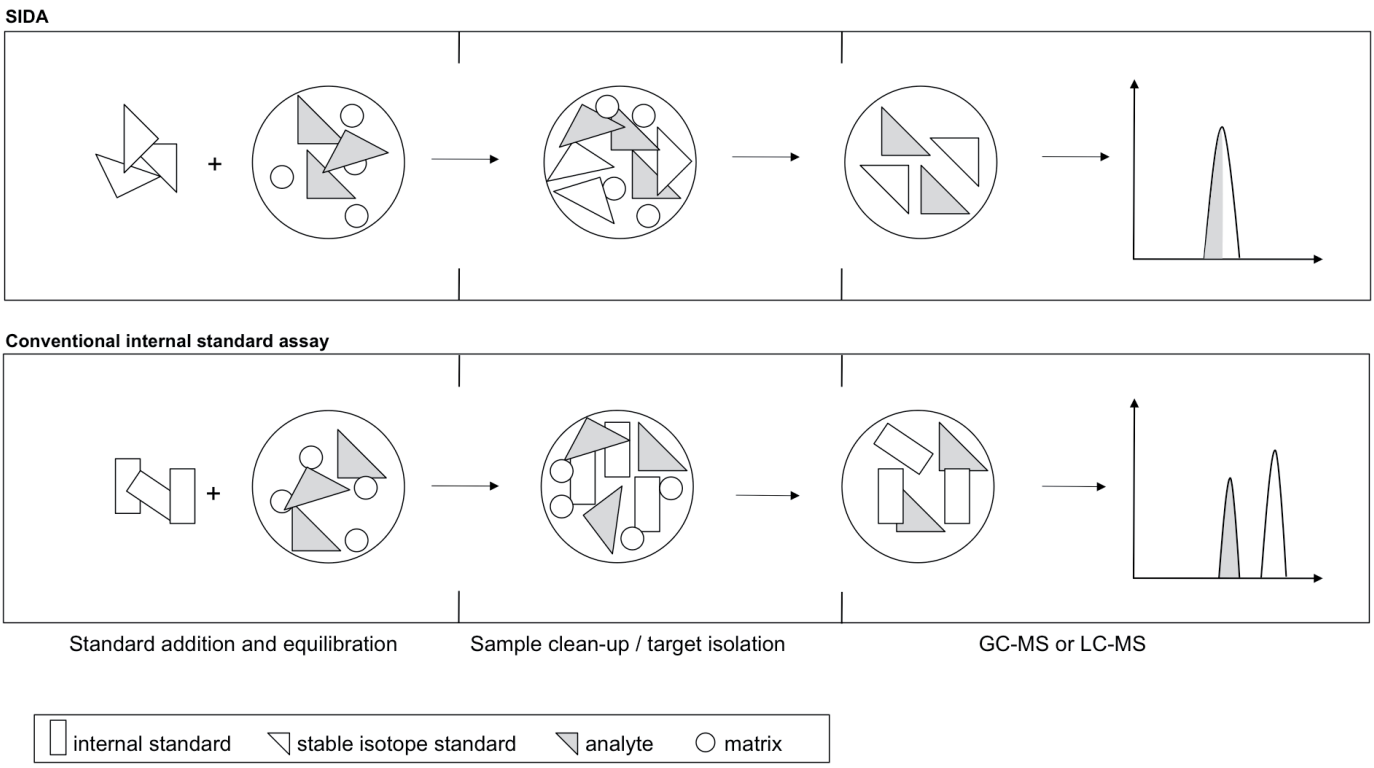

Fig. 2 **SIDA is a special case of an internal standard assay. The identical structure of analyte and standard guarantees an equal behavior during sample preparation, chromatography and MS. In contrast to conventional internal standard assays loss of isotope standard equals the loss of analyte and vice versa**

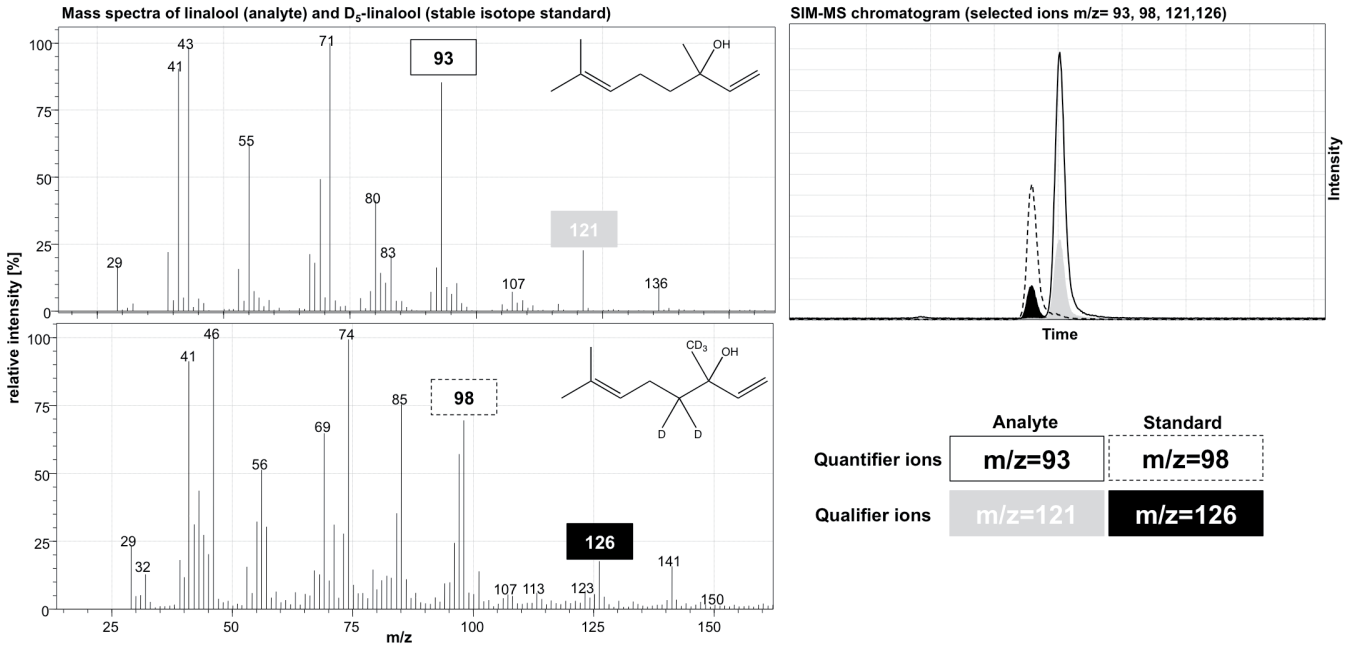

Fig. 3 **Mass spectra of linalool and D_5-linalool show corresponding peaks appearing at shifted m/z. In the SIM-MS chromatogram those characteristic peaks are the selected ions. M/z = 93 and m/z = 121 represent the analyte, m/z = 98 and m/z = 126 the isotope standard. The intensity of these ions indicates the ratio of both isotopologues. In order of IE caused by D_5-labeling standard elutes prior to the analyte**

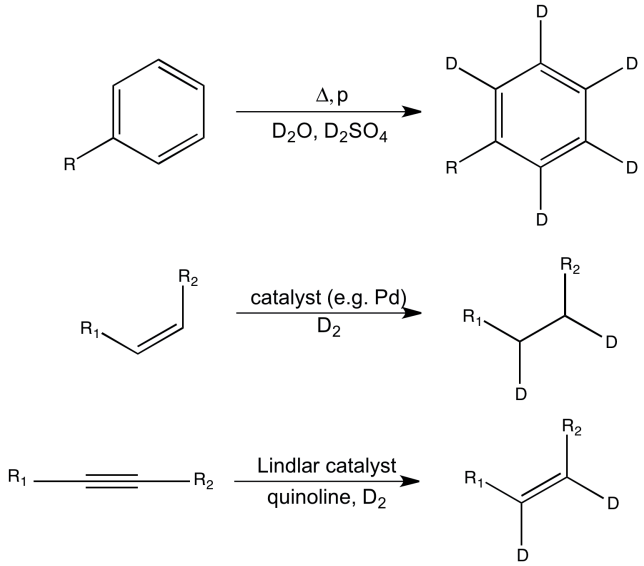

Fig. 4 **Selected examples for simple deuteration reactions. D enriched chemicals are comparably cheap, parameters of deuteration reactions are generally mild**

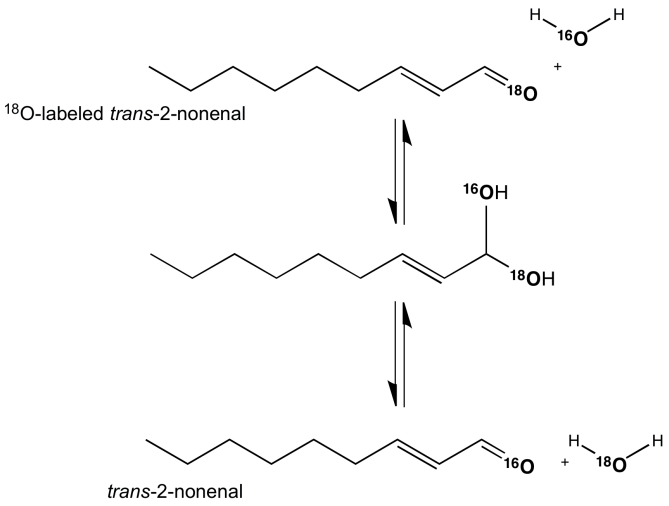

Fig. 5 **The ^{18}O-label of *trans*-2-nonenal is exchanged by water addition and elimination. In beer (or other slightly basic/acidic media) ^{18}O-labeling of carbonyls is generally improper for SIDA, false quantification and misinterpretations are very likely**