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GC-MS-IRMS

Standard Operating Procedure for $\delta^{13}C$, $\delta^{2}H$ and $\delta^{18}O$ analysis of vanillin in vanilla extracts

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Introduction

Vanillin can be extracted from vanilla beans through a process that involves curing and fermentation. This traditional method yields a natural vanillin extract, which is often used in food products. Due to the limited availability and high cost of natural vanilla, synthetic vanillin is produced through chemical synthesis and used as a more affordable alternative. Vanillin derived from different sources can have distinct isotopic composition that can be investigated by compound-specific isotope analysis via GC-IRMS.[1-4] Here we present detailed instructions for measuring $\delta^{13}C$, $\delta^{2}H$ and $\delta^{18}O$ of vanillin in vanilla extracts. This 3-dimensional isotope signature helps to distinguish between natural and synthetic and many nature-identical sources of vanillin.

Equipment

All measurements are performed using a Thermo Scientific™ GC IsoLink™ II IRMS System on-line coupled to a Thermo Scientific™ ISQ™ Series Single Quadrupole MS. This analytical setup provides a routine methodology for simultaneous acquisition of mass spectra (compound identification and quantitation) and carbon, hydrogen, nitrogen, and oxygen stable isotope analysis based on combustion/reduction and high temperature conversion (HTC)/pyrolysis methodology.

Analytical material is injected into a Thermo Scientific™ TRACE™ Series GC via a TriPlus™ RSH Series Autosampler. The analytes are separated in a GC column followed by a split of the carrier gas in Micro Channel Device (MCD) in two directions: (i) a minor part is introduced into the single quadrupole MS where the analytes get identified based on a mass spectrum, (ii) a bigger part of the sample gets into the conversion reactor where the analyte is converted into a simple gas (CO_2, H_2, CO), followed by a trap for water removal. A second MCD allows for installation of a ^{13}C combustion and ^{2}H high temperature conversion reactor in parallel, or installation

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of ^{18}O high temperature conversion reactor with an auxiliary gas. The resulting analyte gas is introduced in the Thermo Scientific™ DELTA™ Series IRMS via ConFlo IV™ Universal Interface for isotope ratio measurements (Figure 1). Despite the flow split between MS and IRMS, there is no sensitivity loss in IRMS because of the open split design in the ConFlo IV Universal Interface.

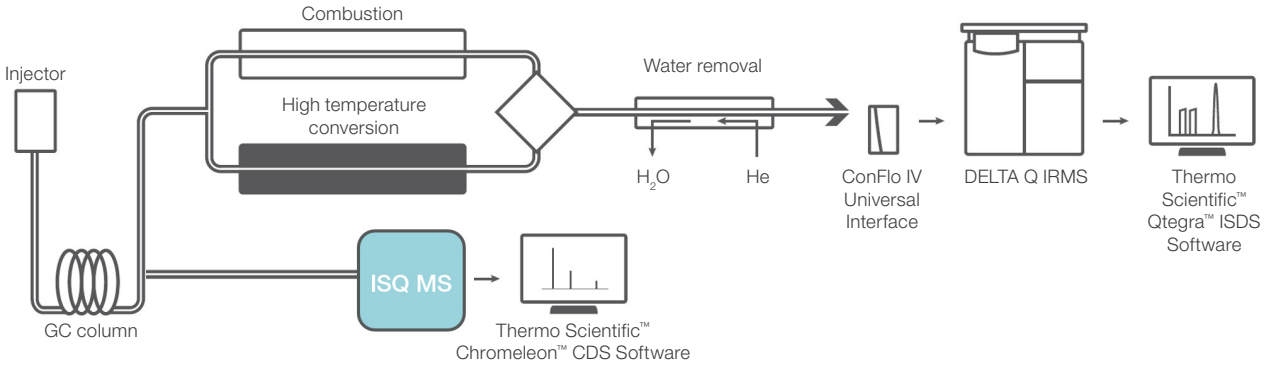

Figure 1. GC-MS-IRMS System workflow.

Analytical setup

Detailed analytical setup for the TriPlus RSH Series Autosampler, the iConnect SSL Injector Module, TRACE Series GC and ISQ Series MS is listed in Table 1.

Table 1. Autosampler, gas chromatographer and MS analytical setup

Triplus RSH Series autosampler parameters		iConnect SSL Injector Module parameters		TRACE Series GC parameters	
Thermo Scientific™ Fixed Needle Autosampler Syringe, 10 µL, 26 s G, Cone, 57 mm ND10 Screw Neck Vial 1,5 mL 32 x 11.6 mm ND10 Screw Cap (Black) 10 mm, Silicone/PTFE Septa Micro-insert, 0.1 mL, 29x5.7 mm		Thermo Scientific™ LinerGOLD Splitless GC Liner, Singe Taper, Quarty Wool P/N 453A1925-UI		Thermo Scientific™ TraceGOLD™ TG-5MS Column, 30 m, 0.25 mm, 0.25 µm P/N 26098-1420	
Injection type	Liquids	Injection temperature	260 °C	Temperature	80 °C
Sample volume	1-3 µL	Inlet module and mode	SSL, splitless	Hold time	1 min
Pluger strokes	3	Split flow	150 mL/min	Rate	20 °C/min
Air volume	1 µL	Splitless time	0.5 min	Temperature 2	180 °C
Filling volume	3 µL	Purge flow control	active	Hold time	0 min
Sampling depth in vial	28 mm	Constant septum purge	5 mL/min	Rate	70 °C/min
Pre-injection delay	0.5 s	Gas saver flow	15 mL/min	Temperature 3	330 °C
Post-injection delay	1.5 s	Gas saver time	5 min	Hold time	6.7 min
Injection depth	45 mm	Carrier gas, flow, Constant Flow	He, 1.3 mL/min	GC run time	15 min
Penetration speed	50 mm/s	ISQ Single Quadrupole MS			
Injection speed	55 µL/s	MS transfer line temp. (°C)		280	
Sample pullup speed	1 µL/s	Ion source temp (°C)		280	
Delay after plunger strokes	0.2 s	Ionnization mode		EI	
Viscosity delay	0.2 s	Run completion (min)		15	
Bubble elimination pullup	2 µL/s	Scan time (min)		4	
Delay between strokes	0.1 s	Mass list or range (amu)		35-350	
Solvent filling speed	2 µL/s	Dwell or scan times (sec)		0.2	
		Tune file name		Last saved	
		Emission current (µA)		20	

In case of some contaminants interfering with the vanillin peak, the GC method would need to be adapted, and depending on the composition of the vanillin extract, a medium/high polarity column might be required to achieve optimal peaks resolution.

Based on the oven temperature program (Table 1) and volatility of the solvent (MTBE), the backflush function of the GC IsoLink II Conversion Interface was switched off at 300 s, allowing the compounds of interest eluting from the column to be transferred into the conversion reactor.

Detailed information on each configuration and reactor setup is provided in the Chapter 'Instrument preparation and maintenance procedures'.

Analytical materials

Good laboratory practice mandates that scientific results are reported relative to recognized reference materials and traceable to internationally agreed scale; the primary reference materials are inorganic compounds whose chemical elemental composition does not match that of organic compounds and materials, the secondary reference materials are natural or synthetic compounds that have been carefully calibrated relative to the primary.[5] These reference materials are not recommended for daily use as they are in short supply but are primarily used to calibrate in-house reference materials for everyday use for data normalization and quality assurance. In-house reference materials should also be chemically similar to the samples, according to the Principle of Identical Treatment (PIT).[6]

Different pure vanillin standards were selected to be used as in-house reference materials; they are all chemically identical to the samples target compound and their isotopic composition (which was determined by EA/HTC – IRMS) are bracketing those of the samples. Table 2 shows an example of recommended reference materials for this application. For vanillin extracts analysis, unknown samples are prepared and measured in sequences along with in-house reference materials and quality control (QC) materials, which are used to evaluate the analytical performance of the measurement. The QC material must have matrix similar to the samples, requiring the same extraction and cleaning procedure for GC analysis. The best practice is to obtain a material from interlaboratory tests with a known isotopic composition. Alternatively, the isotopic composition of QC can be determined by GC-IRMS using in-house reference materials.

Table 2. Pure vanillin from different origins suggested as reference materials

	Min		Max	
	Material	Expected value	Material	Expected value
$\delta^{13}C$	Vanillin ex-ferulic acid	-35 ‰	Vanillin ex-glucose*	-13 ‰
$\delta^{2}H$	Vanillin ex-ferulic acid	-180 ‰	Synthetic Vanillin	+70 ‰
$\delta^{18}O$	Synthetic vanillin**	+1 ‰	Vanillin ex-glucose	+ 16 ‰

***C4 plant**
**** some of the synthetic vanillin have values around 1 ‰, others around 8 ‰**

Analytical materials preparation

Powders (samples and reference materials)

1 mg powder is transferred into a vial and 1 mL Methyl tert-butyl ether (MTBE, anhydrous 99.8% or better) is added. The mixture is homogenized for a minute on a Vortex Mixer. The sample can be stored up to 6 weeks in the refrigerator until measurement. The reference material is diluted as described in Table 3.

Table 3. Suggested concentration of pure vanillin for determination of the carbon, hydrogen and oxygen isotope signature

Isotope signature	Vanillin concentration (mg/mL)	Injected element in 1 µL (ng)	Average Peak Amplitude on IRMS (V)
$\delta^{13}C$	0.2	126	2-3
$\delta^{2}H$	1.0	52	3-4
$\delta^{18}O$	0.5	157	3

Vanilla extracts

1 mL of the vanilla extract is transferred into a transparent vial and 1 mL MTBE (anhydrous 99.8% or better) is added. The mixture is homogenized for a minute on a Vortex Mixer. The top layer (organic phase) is transferred into another vial (e.g. 2 mL tube). This extraction is repeated by adding another 1 mL MTBE to the vanilla extract, shaking for about 1 min and transferring the organic phase into the vial, pooling the extractions.

The extracts can be stored up to 6 weeks in the refrigerator until measurement.

Sample extracts with unknown content are analyzed on a GC-MS or an alternative GC detector prior to measurement on the GC-MS-IRMS to estimate the vanillin concentration. According to this estimation, samples are diluted with MTBE to achieve similar peak height to the in-house reference materials.

Analytical workflow

In-house reference materials should be measured both at the beginning and the end of the sequence. Samples should be measured at minimum in duplicates, preferably in triplicates, to ensure precision control. Every 6th sample at a minimum should be a quality control. Typically, the memory effect is negligible for $\delta^{13}C$, $\delta^{18}O$ and for $\delta^{2}H$ measurement, but for hydrogen isotope analysis it is recommended to analyze samples in triplicates. The $\delta^{18}O$ measurement requires drift correction using the reference material which is injected every third measurement. The system must be conditioned with 2-4 conditioning injections before starting a measurement sequence. A typical measurement sequence is described in Table 4.

Table 4. Example sequence structure for measuring $\delta^{13}C$, $\delta^{2}H$ and $\delta^{18}O$ isotope ratios in vanillin

$\delta^{13}C$		$\delta^{2}H$		$\delta^{18}O$	
Item	repetition	Item	repetition	Item	repetition
Conditioning injections (Reference material)	2-4	Conditioning injections (Reference material)	2-4	Conditioning injections (Reference material)	2-4
Reference 1	2	Reference 1	3	Reference 1*	2
Reference 2	2	Reference 2	3	Reference 2	2
Sample 1	2	Sample 1	3	Sample 1	2
Sample 2	2	Sample 2	3	Sample 2	2
Sample 3	2	Sample 3	3	Reference 1*	1
Sample 4	2	Sample 4	3	Sample 3	2
Quality control	2	Quality control	3	Sample 4	2
Sample 5	2	Sample 5	3	Reference 1	1
Sample 6	2	Sample 6	3	Quality control	2
Sample 7	2	Sample 7	3	Sample 5	2
Sample 8	2	Sample 8	3	Reference 1*	1
Reference 1	2	Reference 1	3	Sample 6	2
Reference 2	2	Reference 2	3	Sample 7	2
				Reference 1*	1
				Sample 8	2
				Reference 2	2
				Reference 1*	2

*used for drift correction

Instrument preparation and maintenance procedures

Table 5 describes typical preparation and maintenance procedures for GC-C-IRMS System used for vanillin authenticity analysis.

Table 5. Instrument preparation and maintenance procedures for GC-MS-IRMS System

	Action	Frequency
Syringe	Prime manually with methanol	Before each sequence
Liner	Clean and deactivated	Replacement every max. 100 injections
GC Column	Clean up: solvent injection with same GC method but adding 30 min to final temperature hold time.	After each sequence
Gas Saver	Not recommended for ^{18}O analysis	NA

Table 5. Instrument preparation and maintenance procedures for GC-MS-IRMS System continued

	Action	Frequency
ISQ MS	Daily tune check: leak check/resolution/ sensitivity	Check and record before each sequence
IRMS	Backgrounds check in backflush mode	Check and record before each sequence
IRMS	Stability test (on-off test)	Check and record before each sequence*
IRMS ($^{13}C/^{18}O$ analysis)	Linearity test	Every 1-4 weeks
IRMS (^{2}H analysis)	H_3^+ Factor check and stability	Before each sequence
GC-IRMS	Backgrounds check in straight mode (≤ 100 mV for $^{13}C/^{2}H$ and ≤ 70 mV for ^{18}O).**	Check and record before each sequence

*It is recommended to record in the Logbook the reference peaks amplitude in the on-off test to be used as a reference for the instrument sensitivity.

** For ^{2}H and specially for ^{18}O analysis, having a low air (specifically H_2O/O_2) level in the background is critical to maintain the reactor in good status. It is recommended to maintain the argon level as low as possible. In case of finding high levels of air in the background, it is recommended to reduce the reactor temperature to 400-600 °C until the leak is fixed, and once the reactor is back to measurement temperature, it is recommended to condition it before running samples.

GC-C-IRMS for $\delta^{13}C$ determination

Reactor measurement temperature: 1000 °C

Reactor regeneration procedure:

- Before each sequence: Oxidation 1 hour / Backflush 1 hour / Vent 0.2 min
- Seed oxidation before injection: Seed oxidation 0.2 min / Backflush 0.2 min / Vent 0.2 min
- Standby temperature conditions
  - Standby time ≤7 days: 600 – 1000 °C
  - Standby time ≥ 7 days: 400 °C

NOTE: when running ^{13}C analysis, the HTC reactor heater must be always ON, at ≥ 400 °C.

GC-HTC-IRMS for $\delta^{2}H$ determination

Reactor measurement temperature: 1420 °C

Reactor conditioning:

- Before each sequence: 2-4 reference material injections, as outlined in Table 4
- Standby temperature conditions: to extend the reactor and heater lifetimes, it is recommended to cool down the HTC reactor for Standby
  - Standby time ≤ 7 days: 600 °C
  - Standby time ≥ 7 days: 400 °C

NOTE: when running ^{2}H analysis, the combustion reactor heater must be always ON, at ≥ 400 °C.

GC-HTC-IRMS for $\delta^{18}O$ determination

Initial leak test and transfer test must be always performed in a **cold** reactor (maximum 400 °C). It is very important to prevent O_2 and H_2O entering the reactor when it is hot.

- Auxiliary gas flow 0.6 – 0.8 mL/min. This flow was calculated by subtracting the flow measured in straight mode at the reactor exit without auxiliary gas from the flow measured with auxiliary gas

Reactor measurement temperature: 1280 °C

Reactor conditioning:

- New reactor initial conditioning: reactor at measurement temperature / Auxiliary gas On / LF disconnected / System in straight mode overnight
- Reactor conditioning for measurements:
  - 1 µL cyclohexane injected in Split mode (Spilt ratio 1:20) keeping the GC IsoLink II in straight mode and LF disconnected. Background stabilization time: 2 hours
  - After ca. 8 hours measuring samples, it is necessary to recondition the reactor following the same procedure

    NOTE: The CO background is several 100 mV and slowly decreases throughout the measurement sequence. The effect is corrected by drift correction.
- Standby Conditions: maintain the GC IsoLink II conversion interface with the Backflush closed and the LF disconnected. For ^{18}O analysis the Gas Saver option must be OFF in the GC standby method

  Temperature:
  - Standby time up to two days: maintain 1280 °C
  - Standby time 2-7 days: 600 °C
  - Standby time ≥ 7 days: 400 °C

Results

The ISQ MS chromatogram helps to identify the vanillin peak on the IRMS chromatogram. The peak shape and mass spectrum reveal possible impurities in the peak (Figure 2).

Exemplatory chromatograms of the IRMS are shown on Figure 3, Figure 4, Figure 5.

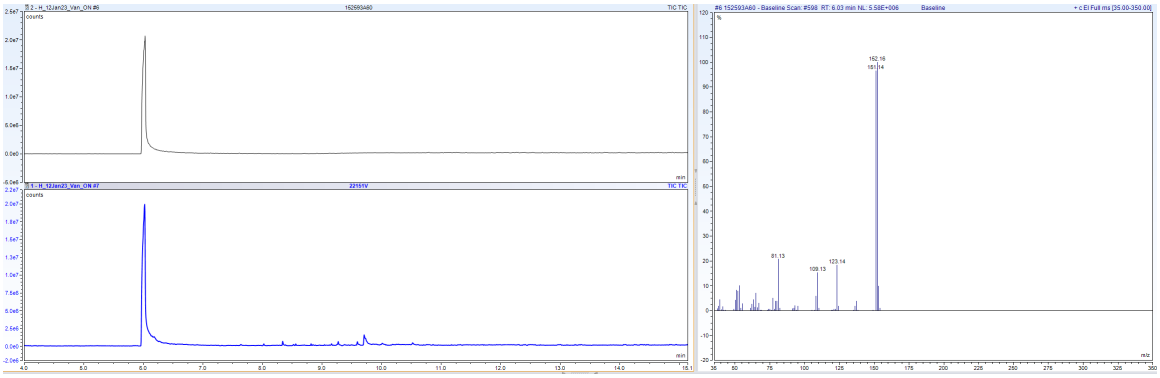

Figure 2. Example of an ISQ MS chromatogram and the mass spectrum of the vanillin extract.

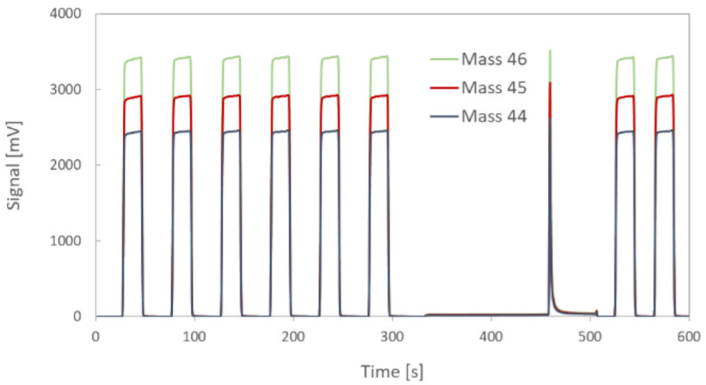

Figure 3. Example of an IRMS chromatogram for $\delta^{13}C$ measurement of vanillin.

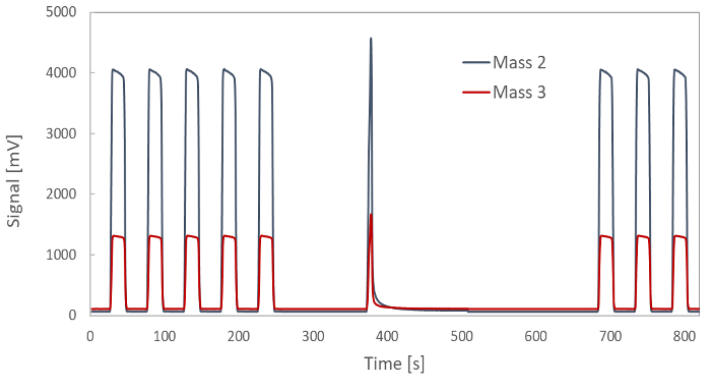

Figure 4. Example of an IRMS chromatogram for $\delta^{2}H$ measurement of vanillin.

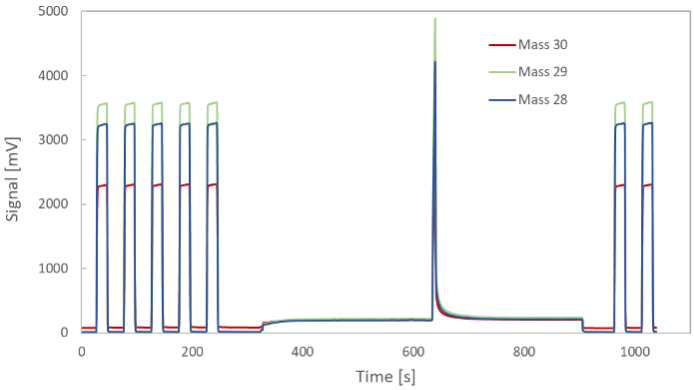

Figure 5. Example of an IRMS chromatogram for $\delta^{18}O$ measurement of vanillin.

The evaluation and post processing of the isotope values is described in detail in FIRMS guidelines (*Dunn and Carter, 2018*). Here we highlight some points especially important for GC-IRMS measurement.

Normalization

2-points normalization is mandatory. However, in case of carbon measurement the scale contraction is usually very small (approx. ± 1-2%); thus, 1 point calibration would be possible. Alternatively to the two points calibration, a three (or more) points linear regression can be used for normalization of the data. In this case the quality of the correction can be evaluated by the integrity of the regression line.

Drift correction

By comparison of the offset values of the reference materials at the beginning and the end of the sequence it can be decided if drift correction is needed. In the case of hydrogen and carbon measurement, if the conditioning of reactors was correct, drift correction is usually not needed. In the case of $\delta^{18}O$ measurement a drift correction is needed almost in all cases.

Memory correction

The memory effect is usually negligible; thus, its appearance indicates that the system should be investigated. In most cases replacement of the liner and trimming the column head eliminates the problem. Affected measurement sequences can usually still be used by discarding the first result of repeated measurements of the same sample.

Quality Control

The usual standard deviation of repeated measurements for one sample are 0.1-0.2 ‰ for $\delta^{13}C$, 1-2 ‰ $\delta^{2}H$ and 0.3-0.4 ‰ for $\delta^{18}O$ isotope values, respectively. Aggregated measurement error for one sequence, which includes the reptations error of the sample and the error of referencing, are typically 0.3-0.4 ‰ for $\delta^{13}C_{VPDB}$, 4 ‰ for $\delta^{2}H_{VSMOW}$ and 0.8-1.0 ‰ for $\delta^{18}O_{VSMOW}$. The analytical performance of the measurement can be monitored by quality control charts. In these charts the calculated delta value of the quality control material is compared to the accepted value (Figure 6). For the acceptance or refusal of the measured values, the Westgard rules (*Dunn and Carter, 2018*) should be applied.

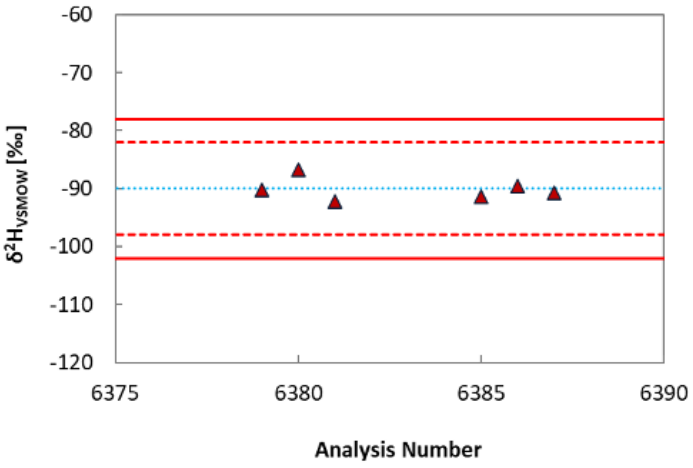

Figure 6. Example for accuracy check of the quality control sample. The normalized QC values are represented with triangles. Warning limit (±2σ) and control limit (±3σ) are depicted with dashed and thick lines, respectively. Accepted error (σ), in this case 4 ‰.

In case of proper maintenance of the GC-IRMS system a robust long-term performance can be achieved, as shown in Figure 7 for a period over 3+ years.

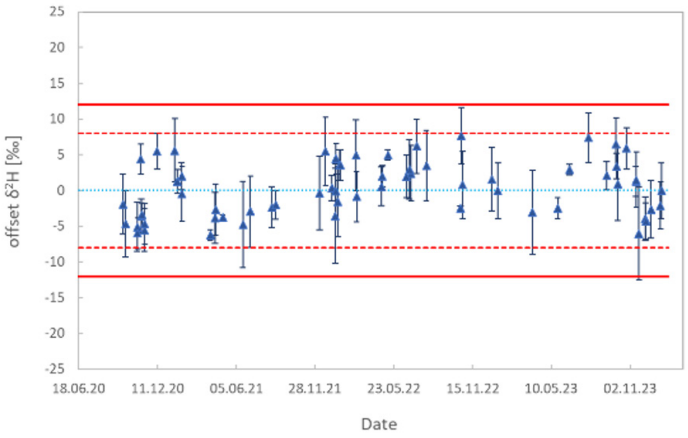

Figure 7. Long term accuracy of the $\delta^{2}H_{VSMOW}$ values of the quality control vanillin. The triangles and error bars represent the mean offset of the standard deviation of QC materials for a measurement day. Warning limit (±2σ) and control limit (±3σ) are depicted with dashed and thick lines respectively. Accepted error (σ) in this case is 4 ‰.

Regular inter-laboratory testing is mandatory for the final proof and control of accurate measurement.

Conclusions

We present standard operating procedure for $\delta^{13}C$, $\delta^{2}H$ and $\delta^{18}O$ analysis of vanillin in vanilla extracts with conventional split/splitless injector. By using a hybrid Thermo Scientific GC-MS-IRMS system, structural information and isotope ratio data of individual peaks can be obtained from a single injection. By adhering to here described procedures for analysis setup, instrument maintenance and data evaluation and normalization, highly accurate and precise isotopic data for vanillin can be obtained.

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