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EA-IRMS

Workflow for the NCS analysis of collagen: instrument setup and measurement strategies

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Introduction

Isotope fingerprints of nitrogen, carbon and sulfur are a powerful tool to interpret the diet and mobility of populations in archeological and ecological studies. Typical sample materials are bone collagen or dentine. Sulfur isotope analyses of these materials can be very challenging due to extremely small weight percent values (often ≤ 0.3 weight %); especially taking into consideration that these samples are often rare and available in small amounts, making it difficult to have enough material for $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ analyses.

The Thermo Scientific™ EA IsoLink™ IRMS system provides a solution for isotope analysis of low concentrations of sulfur as a part of sequential NCS analysis using the High Sensitivity Method for sulfur[1]. Implementation of this optimized NCS workflow reduces the required amount of sulfur for triple analysis to 3-5 µg, enabling NCS isotope analysis on smaller sample amounts than before. Three µg of sulfur corresponds e.g. to ~1-3 mg collagen assuming a weight % range of 0.1-0.3.

The principle of the High Sensitivity Method for sulfur is to preconcentrate SO_2 gas, generated in the sample combustion, on the GC column. This is achieved by proprietary Helium management system and the temperature-ramped GC oven allowing optimal separation of gases and sharp peak shapes. The ideal timing of Helium Management (HeM) Module activation, as well as the flow reduction degree and the use of a temperature ramping GC Oven to sharpen the peak shape, needs to be determined during method development.

Reducing the amount of SO_2 sample gas to this level requires a new level of carry-over suppression, which needs to be encountered with a strict hardware optimization for sulfur and appropriate maintenance of the system.

In this document we describe the Thermo Scientific™ High Performance Sulfur Kit that provides hardware optimization, and show how to optimize the NCS workflow for collagen analyses focusing on: instrument maintenance, SO_2 preconcentration, and techniques to test and maintain SO_2 carry-over suppression.

Equipment

Instrument

NCS isotope fingerprints of collagen were analyzed using the EA IsoLink IRMS System that includes the Thermo Scientific™ Flash IRMS Elemental Analyzer (EA) CNSOH with NCS separation column, temperature-ramped GC oven, and the Thermo Scientific™ MAS Plus autosampler connected via a Thermo Scientific™ ConFlo IV Universal Interface to a Thermo Scientific™ DELTA™ Q IRMS. The instrument is operated using the Thermo Scientific™ Qtegra™ Intelligent Scientific Data Solutions (ISDS) Software, with all isotope measurements carried out in continuous flow mode.

The Flash IRMS EA is equipped with the High Performance Sulfur Kit (PN: BRE0082748) that includes:

- Sulfinert®-coated HeM Module
- An extra-long water trap with Magnesium (Mg) Perchlorate filling
- Filters before the needle valve of the HeM Module
- Additional Sulfinert® capillaries

Hardware optimization

The NCS analyses must be performed on a single reactor setup (using a tungsten oxide/copper (NCS) reactor). If an EA IsoLink CN(S) system with double reactor setup is used, the second reactor must be bypassed.

SO_2 carry-over suppression is critical for NCS analysis of small sulfur amounts using the High Sensitivity Method for sulfur and requires hardware optimization. The High Performance Sulfur Kit provides optimized hardware components for the Flash IRMS EA, as depicted in Figure 1. For this analytical setup, particular components in the Flash IRMS EA unit need to be replaced, including the existing HeM replacement by a SulfinertR-coated HeM. Sulfinert® coating is inert with respect to sulfur compounds and is advantageous for the SO_2 analysis. The extra-long water trap from the High Performance Sulfur Kit is installed in the left compartment of Flash IRMS EA, close to the combustion reactor. This supports trapping water early in the flow path, reducing the SO_2 – H_2O interaction (SO_2 is hygroscopic) and the complete transport of SO_2 through the system. A longer water trap with more Mg Perchlorate filling is reducing the likeliness of a water breakthrough (and an increase of the water background in the whole system) which can affect the analytical performance of sulfur analysis over time. Additional filters are installed in front of the needle valves of the HeM Module.

The pressure gauge for SO_2 of the ConFlo IV Universal Interface is heated. The source and needle valve heaters of the IRMS need to be turned ON.

For optimal analytical performance it is necessary to perform regular EA IsoLink System maintenance procedures as described in the following chapter.

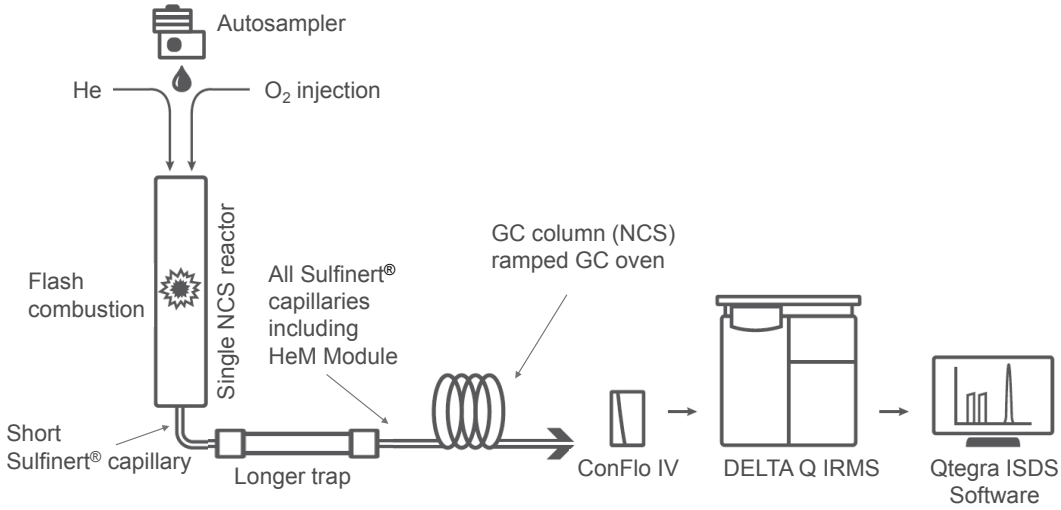

Figure 1. Schematics of the instrument setup: single reactor setup (NCS), longer water trap close to the combustion reactor, Sulfinert® capillaries, Sulfinert®-coated HeM Module, NCS GC column is installed in the temperature-ramped GC oven unit

Instrument preparation and maintenance

Note: Detailed information on the maintenance procedures mentioned, including safety measures, can be found in the respective EA IsoLink Operating Manual and DELTA Q IRMS Operating Manual.

A requirement for the successful application of the High Sensitivity Method for sulfur is a leak-tight, clean (with respect to deposits) EA IsoLink System with accurately set up flows and a well-tuned and calibrated IRMS. The following system preparation and maintenance steps are beneficial for maintaining sulfur carry-over suppression and increasing the GC column separation and pre-concentration performance:

1. Turn off the temperature-ramped GC oven whenever the carrier flow is stopped (i.e., when replacing the reactor or the water trap) to reduce the temperature and avoid air contact with the hot column.

   This will preserve the separation capabilities of the GC column.

2. After replacement of any component of the system the leak test must be performed to ensure that the system is leak tight.

3. When replacing the NCS reactor, it is recommended to also clean the connector at the bottom with a lint free tissue and/or ethanol and regularly replace the Sulfinert® capillary piece between the NCS reactor and the water trap (recommended is min. every 5th reactor exchange).

   Combustion deposits in these spots can be formed which can enhance sulfur carry-over; removing them supports the suppression of SO_2 carry over.

   If thorough cleaning of the connector is required at any time, rinse with dichloromethane, then with methanol and dry in an oven.

4. The first heating of the new NCS reactor must be executed slowly at the rate 10 °C steps per 180 s starting at 400 °C, maintaining the He carrier flow at 50 mL/min (HeM Module setting: V2 closed), the temperature-ramped GC oven at set point 2 (240 °C) and letting it condition overnight.

   While the newly installed NCS reactor is heated for the first time it is recommended that the temperature-ramped GC oven is heated up – so as to not retain any components that might be created during the NCS reactor heating process.

5. The next day, cool down the reactor to 600 °C and set the temperature-ramped GC oven at the set point 1 (60-70 °C), reduce the carrier flow to 10 mL/min and, once the GC oven reaches the set point 1, turn it off and replace the water trap filled with Mg Perchlorate. Clean the connectors of the water trap with a lint free tissue and/or ethanol.

   This step is performed after the first NCS reactor heat-up to remove any components that might accumulate in the trap during the heating process. For SO_2 measurements, it is recommended to exchange the water trap with each new NCS reactor replacement.

6. After the water trap replacement, turn the temperature-ramped GC oven on and test the system for leak tightness, slowly heat up the NCS reactor (10 °C steps per 180 s) maintaining the He flow at 50 mL/min (HeM Module setting: V2 closed).

7. Once the instrument is set to operating conditions, it is recommended to check the carrier flow of the Flash IRMS EA: it can be measured at the vent tube of the pre-split of the ConFlo Universal Interface; target in this location: 42 mL/min).

   Occasionally, it is also a good practice to check the split flow of HeM valve V2 (can e.g. be measured at the autosampler flush; target in this location: 200 mL/min assuming a carrier flow of 180 mL/min and reference flow of 70 mL/min).

8. DELTA Q IRMS and ConFlo IV Universal Interface: Check the system suitability (sensitivity and stability) before the measurement of each sample using the N_2, CO_2 and SO_2 reference gases (if required re-tune using e.g. the Autofocus Module of the TuneBook for optimization). Perform an N_2-CO_2 jump re-calibration (and CO_2-SO_2 if the fast jump is used for switching between these gas configurations) and check the instrument stability running “jump on/off tests”, i.e., running several reference gas pulses of each reference gas (first N_2, then CO_2 and then SO_2) in one run.

9. It is highly recommended to schedule 2 hours bake out of the GC column after the sample sequence for clean-up (recommended number of samples per sequence: 64). When the system is in Standby for more than 2 days, it is also recommended to do a short column clean up (i.e., 30 minutes) before analyzing samples. For good quality results, not more than 180-200 collagen samples should be analyzed on one NCS reactor.

10. For the Standby setup, do not reduce the EA IsoLink System flow below 30-50 mL/min (HeM Block setting: V2 closed), to keep the GC column well maintained and the SO_2 carry-over condition low. For Standby periods above 5 days it is recommended to reduce the reactor temperature to 600 °C.

Analytical setup

The Flash IRMS EA method for the simultaneous analysis of $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ of collagen (temperature and flows; see Table 1) is identical to a classical NCS analyses. Optimization of the timing and duration of the oxygen injection for the system is also an important first step to ensure a complete combustion for collagen analyses (parameters: sampling delay & oxygen injection end; for more information see the respective EA IsoLink Operating Manual).

The acquisition method differs from other NCS analyses as it requires the High Sensitivity Method for sulfur. The acquisition method features are: the preconcentration of SO_2, peak-sharpening of N_2, CO_2 and SO_2 and optionally cleaning steps within the method.

Preconcentration of SO_2 with High Sensitivity Method for sulfur

The principle of the High Sensitivity Method for sulfur is to preconcentrate the SO_2 gas, generated in the sample combustion, on the GC column by closing the V2 in the HeM Module and reducing the He carrier flow before the sample gas is split in V2. When reducing the carrier gas flow, the amount of He becomes less, resulting in a higher concentration of SO_2. For a successful preconcentration of sulfur on the column, the timing and the degree of flow reduction using the HeM Module are crucial parameters.

In the example Timeline in Figure 2, the EA is started at 30 s, which means that the oxygen injection is started at 30 s and the sample will drop after the passing of the Sampling Delay (10 s) and the autosampler rotation (~2s). Hence, the sample combustion takes place at 42 s. The generated sample gases travel quickly and arrive at the HeM module some seconds later. Before the SO_2 arrives at V2, the HeM needs to be activated (V2 off + flow reduction) to facilitate preconcentration. If the HeM is activated too early, it will result in a strong peak broadening of all three gases and no sensitivity gain. If the HeM is activated too late, there will be no preconcentration and no gain in sulfur sensitivity. This transfer time needs to be determined by testing and adapted for the individual instruments during the method development. In this study the transfer time was 8 s; the flow was reduced at 50 s, 8 s after the flash combustion (at 42 s).

In principle, the degree of flow reduction scales with the gain of sulfur sensitivity. This was demonstrated by Sayle et al., 2019, by testing flow reduction values between 10-50 mL/min. However, higher degrees of flow reduction create also broader peaks (for N_2 and CO_2), and this might affect the quality of the obtained data. Which flow reduction degree brings the most benefit with respect to sulfur sensitivity without compromising the instrument performance needs to be tested and adapted for the individual instruments during the method development.

In this study the carrier flow was reduced to 40 mL/min. Note that it is important to check the accuracy of the flow rates if a strong carrier flow reduction to e.g. 10 mL/min is intended. The carrier flow should not be lower than 10 mL/min, to ensure that enough He flow is maintaining the ConFlo IV Universal Interface open split, otherwise air can enter the IRMS.

Peak sharpening with the ramped GC oven

Collagen samples have high N/S and C/S ratio and require the usage of adequate dilution settings during the analyses (e.g., N 79 % and C 98 % on 2 mg collagen). However, a column overload effect for carbon can still occur and the flow reduction of the High Sensitivity Method leads to additional peak broadening. This can be addressed by starting the ramping of the temperature-ramped GC oven early, to sharpen the CO_2 peak. Early ramping of the ramped GC oven also results in a retention time shift towards an earlier arrival of the CO_2 peak and SO_2 peak which needs to be considered during the method development (see Figure 2 and Figure 3). The optimal timing of the temperature ramping depends on the separation settings and the performance of the GC column and needs to be determined by tests during the method development. In this study, the temperature ramping was started at 185 s.

Cleaning steps to suppress SO_2 carry-over effects

There are also applicative strategies which can result in an additional SO_2 carry-over suppression. Those can be used to further improve the instrument performance with respect to SO_2 analyses.

Extending the heating interval of the ramped GC oven (from e.g. 350 s to 500 s) in the timeline (acquisition method) can be beneficial as this is conditioning/cleaning the GC column longer/more efficiently between the individual runs. However, this leads to an extension of the acquisition time to accommodate also the cool-down of the GC column before the next run (see Figure 2, Figure 3).

It is also beneficial to execute 1 or 2 'empty' runs (without injection of capsules or oxygen) between different samples, as cleaning steps. See chapter *Results>SO_2 carry over assessment* for more information.

Flash IRMS EA and the acquisition method

The parameters and the acquisition method for the NCS collagen measurements conducted for this study are shown in Table 1 and Figure 2. The method was developed for an EA IsoLink CNSOH with Thermo Scientific™ smartEA option and operated by Qtegra ISDS Software. Adjustments to the parameters shown in Table 1 and Figure 2 may be required to achieve optimal chromatography and analytical results on other systems. An example EA IsoLink IRMS chromatogram of USGS89 is shown in Figure 3.

Table 1: Parameters of the Flash IRMS EA method for sequential NCS analysis of collagen using the High Sensitivity Method for sulfur

Parameter	Value (unit)
Combustion furnace	1020 °C
GC oven (containing the TCD)	70 °C
Temperature-ramped GC oven	Set point 1 (70 °C), Set point 2 (240 °C)
Carrier flow	180 mL/min
Reference flow	70 mL/min
Oxygen flow	250 mL/min
Sampling delay	10 s
Oxygen injection end	5 s

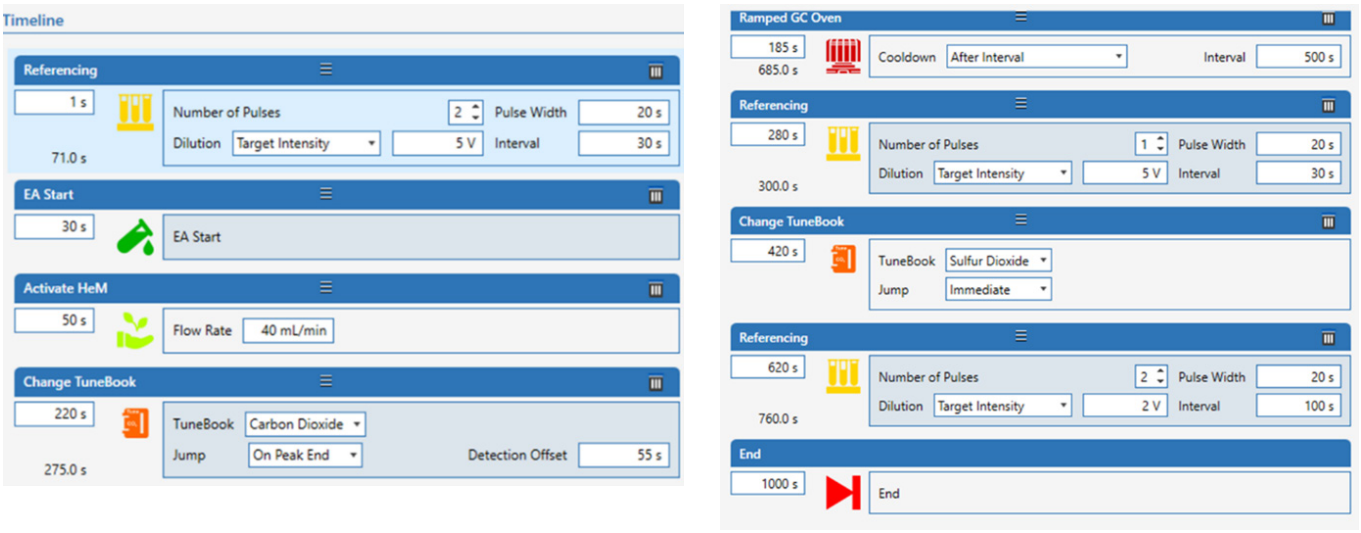

Figure 2. Example timeline of Qtegra ISDS Software displaying the different acquisition steps which need to be performed for the sequential NCS analysis of collagen using the High Sensitivity Method for sulfur

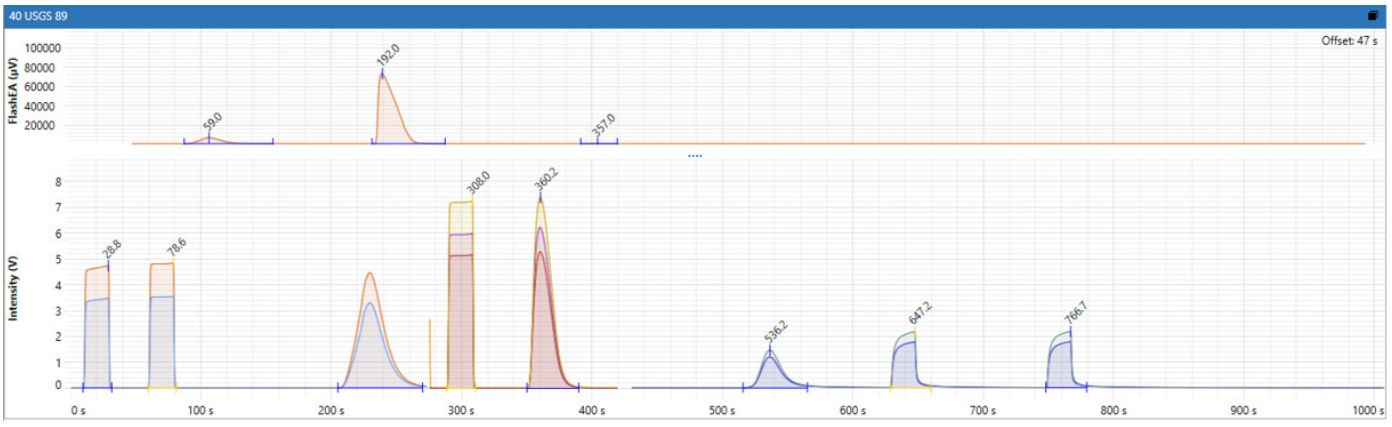

Figure 3: Example chromatogram: USGS 89 (=porcine collagen), m=1.56 mg = 4.2 µg S. SmartEA capillary is shifting the retention times; the gas is arriving later in the open split of the ConFlo IV Universal Interface. Note: shown data is acquired on an EA IsoLink IRMS instrument with an additional resistor in the amplifier used for the cup detecting m/z 66 (which reduces the amplification for this mass).

Results

SO_2 carry-over assessment

To assess the SO_2 carry-over condition of the instrument after hardware preparation and method development, materials with different $\delta^{34}S$ values must be analyzed. SO_2 carry-over is a memory effect: lingering SO_2 gas of sample A is affecting the results of the sample run B and is manifesting as an offset in the data. The larger the difference in $\delta^{34}S$ ($\Delta\delta^{34}S$) between the sample runs, the better the visibility of a possible carry-over effect in the $\delta^{34}S$ results. Hence, it makes sense to analyze materials with a $\Delta\delta^{34}S$ which is comprising the $\delta^{34}S$ of the sample materials to get representative test results for the SO_2 carry-over condition. The isotopic reference material USGS88 (marine collagen from wild-caught fish, certified value: $\delta^{34}S$=17.1 ‰, wt% S: 0.48) and USGS89 (porcine collagen, certified value: $\delta^{34}S$ = 3.89 ‰, wt% S: 0.28) were analyzed in this study to assess the SO_2 carry-over condition of the instrument. At a later stage, different gelatines (fish, porcine, bovine) in-house standards were used for further testing.

The first experiment tested the SO_2 carry-over condition of the instrument for different collagen/sulfur amounts: 4 replicates of USGS88 and USGS89 were analyzed alternatingly, first with 2.5 mg of material (11.5-7 µg S), followed by measurements with 1.5 mg of material (7-4.2 µg S). The sulfur isotope data acquired for USGS88 and USGS89 show a good data repeatability with a measurement uncertainty ≤0.5 ‰ (SD for N = 16; Table 2). The results are indicative of relatively low SO_2 carry-over. There was no strong dependence observed regarding the SO_2 carry-over degree and the sample amount. The experiments were continued with ~1.5 mg of collagen (7-4 µg S) as this amount was sufficient for analysis.

Table 2. NCS isotope data of experiment 1; analysis of the reference materials USGS88 and USGS89

Label	δ^{15}NAir* (‰)	SD	$\delta^{13}C_{VPDB}$ ** (‰)	SD	$\delta^{34}S_{VCDT}$ *** (‰)	SD	N
USGS88	14.96	0.03	-16.06	0.05	17.10	0.26	16
USGS89	6.25	0.06	-18.59	0.09	3.86	0.51	16

*applied corrections: two-point scale corrections using USGS88 and USGS89
**applied corrections: blank corrected and scale corrected with USGS88 and USGS89
***applied corrections: scale corrected with USGS88 and USGS89

The second experiment tested the effect of two 'empty' runs as cleaning steps between different samples. The experiment was executed running duplicates of USGS88, USGS89 and bovine, porcine and fish gelatine in-house standards. The 'empty' runs are runs without capsule and oxygen addition, only temperatures and helium flow settings are executed, baking and flushing the system. Even less evidence of carry-over was observed (see Figure 4) and the measurement uncertainty improved (see Table 3).

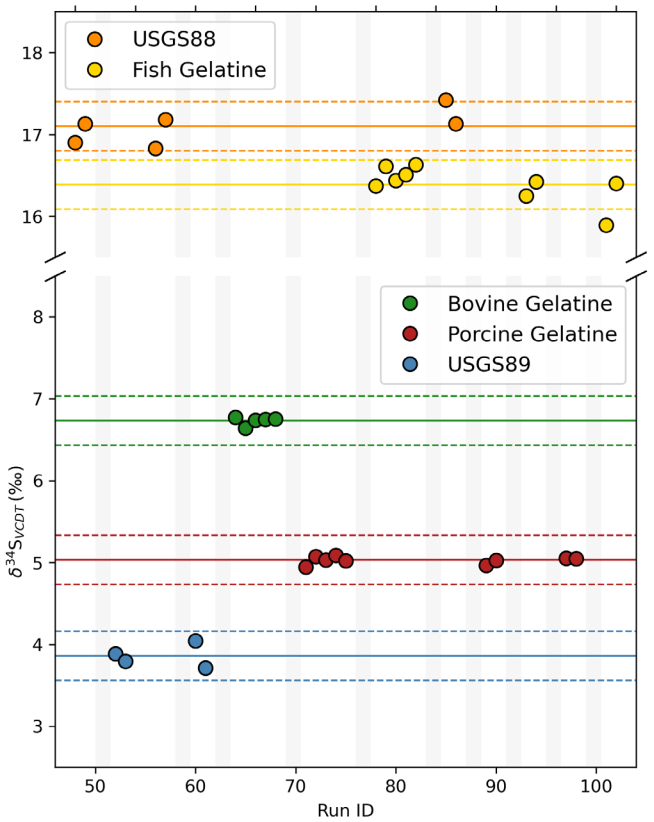

Figure 4: Assessment of sulfur carry-over running 1.5 mg of different collagen materials separated by two 'empty' runs (each grey line illustrates the timing of the two 'empty' runs). The solid lines represent the certified values of USGS88 and USGS89 and for the gelatine in-house standards the determined mean value. The respective dashed lines represent ±0.3 ‰, which is the expected precision for a sulfur-only analysis as reported by Sayle et al., 2019.

Table 3: NCS isotope data of experiment 2; analysis of the reference materials USGS88 and USGS89, and bovine, porcine and fish gelatine in-house standards. The analyses of different collagen materials were separated by two 'empty' runs. Isotopic compositions in light grey cannot be used to assess the accuracy, because those refer to the reference materials which were used for the scale correction.

Label	$\delta^{15}N_{Air}$ * (‰)	SD	$\delta^{13}C_{VPDB}$ ** (‰)	SD	$\delta^{34}S_{VCDT}$ *** (‰)	SD	N
USGS88	14.96	0.05	-16.06	0.07	17.10	0.21	6
USGS89	6.25	0.07	-18.13	0.06	3.86	0.14	4
Bovine gelatine	7.32	0.02	-17.07	0.14	6.73	0.05	5
Fish gelatine	14.13	0.08	-15.67	0.05	16.39	0.22	9
Porcine gelatine	4.87	0.04	-15.30	0.07	5.03	0.05	9

*applied corrections: two-point scale corrections using USGS88 and USGS89
**applied corrections: blank corrected and scale corrected with USGS88 and USGS89
***applied corrections: scale corrected with USGS88 and USGS89

For further simplification of the analytical setup and more productivity, we have tested using one 'empty' run between different samples with the temperature-ramped GC start set at 10 seconds to increase the cleaning efficiency. As demonstrated in Figure 5, one 'empty' run is sufficient to minimize the carry-over effect.

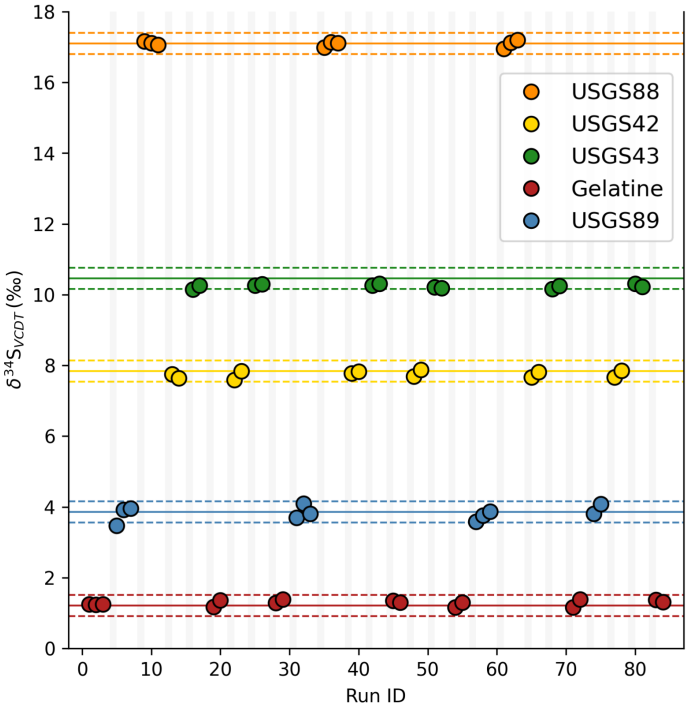

Figure 5: Assessment of sulfur carry-over of different collagen materials separated by one 'empty' run - (each grey line illustrates the timing of one 'empty' runs). The solid lines represent the certified values of USGS88, USGS89, USGS42 and USGS43, and for the gelatine in-house standards the determined mean value. The respective dashed lines represent ±0.3 ‰, which is the expected precision for a sulfur-only analysis as reported by Sayle et al., 2019.

NCS performance assessment

Triplicates of bovine gelatine were analyzed as quality control standards using the method described in the section 'Analytical setup'. Quality control standards are reference materials of known isotope composition which are treated identically to the sample material to check not only the external reproducibility but also the accuracy of the measurement. USGS88 ($\delta^{15}N$: 14.96 ‰, $\delta^{13}C$: -16.06 ‰, $\delta^{34}S$: 17.1 ‰) and USGS89 ($\delta^{15}N$:6.25 ‰, $\delta^{13}C$-18.13 ‰, $\delta^{34}S$:3.86 ‰) as certified reference materials were analyzed together with the bovine gelatine to facilitate a two-point scale correction. A two- or multiple point scale correction using standards with widely different isotopic values is especially important for the $\delta^{34}S_{VCDT}$ determination. Pronounced scale contraction effects are common for sulfur; hence it should be ensured that the isotope composition of the reference materials embraces the isotope composition of the sample which should be scale corrected (for more information see e.g., Dunn and Carter, 2018[2]).

Note, that this can be challenging especially for archaeological collagen samples which show a wide range in $\delta^{34}S$, also strongly negative values; in this case an additional reference material needs to be added with negative sulfur isotope composition to ensure an accurate scale correction. However, this reference material needs also to be similar in its material properties to collagen as standard-sample matrix matching is very important for achieving accurate results for the isotope composition of collagen.

Different sample types might have different sulfur content resulting in varying intensities of sulfur and a linearity correction can be beneficial to achieve accurate results. The linearity effects can be examined by analyzing the quality control standard in different amounts (see Dunn and Carter, 2018 for more information).

The bovine gelatine (~1.5 mg ≈ 248 µg N, 669 µg C, 4 µg S) was analyzed 27 times over two measurement sequences, separated by a reactor exchange, with average $\delta^{15}N_{AIR}$=7.3±0.2 ‰, $\delta^{13}C_{VPDB}$=-17.1±0.1 ‰ and $\delta^{34}S_{VCDT}$=6.8±0.1 ‰ (see Table 3). Two (empty) cleaning runs were setup between different materials to maintain SO_2 carry-over suppression. All data sets were scale corrected using USGS88 and USGS89; in addition to this, the N data (Run B) was drift corrected using USGS89; the C data set was blank corrected and the S data was linearity corrected based on three analyses of different amounts of bovine gelatine executed before the run (1 mg, 1.5 mg and 2 mg). The measurement precision and accuracy are within or close to the system specifications of a classical NCS analyses (without SO_2 preconcentration) of 10 consecutive analyses of sulfanilamide (0.3 mg≈49 µg N, 125 µg C and 60 µg S). Please note that the results of this study don't represent product specifications and are not part of the warranty.

Table 4: NCS isotope ratios data on bovine gelatine, USGS88 and USGS89, separated in Run A and Run B (same analytical setup, different reactors). The analyses of different collagen materials were separated by two 'empty' runs.

Run	Label	Sample type	$\delta^{15}N_{Air}$ (‰)	SD	$\delta^{13}C_{VPDB}$ (‰)	SD	$\delta^{34}S_{VCDT}$ (‰)	SD	N (wt%)	C (wt%)	S (wt%)	N
Run A	USGS88	Delta standard	14.96	0.07	-16.06	0.04	17.10	0.26	16.62	43.08	0.47	12
Run A	USGS89	Delta standard	6.25	0.05	-18.13	0.09	3.86	0.16	15.89	44.23	0.28	12
Run A	Bovine gelatine	QC standard	7.35	0.09	-17.15	0.09	6.79	0.10	16.51	44.60	0.27	12
					Reactor replacement							
Run B	USGS88	Delta standard	14.99	0.25	-16.06	0.07	17.10	0.30	16.78	43.74	0.48	15
Run B	USGS89	Delta standard	6.25	0.19	-18.13	0.14	3.86	0.18	15.77	43.42	0.27	15
Run B	Bovine gelatine	QC standard	7.28	0.27	-17.09	0.13	6.78	0.17	16.18	44.05	0.27	15

Preconcentration and sensitivity gain assessment

A way to compare the sensitivity gain facilitated by the SO_2 preconcentration is: SENS = SO_2 peak area [Vs] / amount of sulfur [µg][1]. In this study, the carrier flow was reduced to 40 mL/min 8 s after combustion, which resulted in a SENS for the analyzed USGS88 of 6.6±0.4 Vs/µg (N=27), USGS89 of 6.0±0.4 Vs/µg (N=27) and bovine gelatine of 6.1±0.5 Vs/µg (N=27). An NCS analyses of sulfanilamide on the same instrument without SO_2 preconcentration yielded 2.2±0.1 Vs/µg (N=10). Hence, we gained approximately 3x the sensitivity by applying the High Sensitivity Technique for sulfur with a flow reduction to 40 mL/min on this instrument. This was sufficient to analyze 3-5 µg of sulfur (corresponds e.g. to ~1-3 mg collagen assuming a weight % range of 0.1-0.3). Please note that these values don't represent product specifications and are not part of the warranty.

Conclusion

The EA IsoLink IRMS System can be used to determine NCS isotope data from one sample drop of 1-3 mg collagen (assuming a weight % range of 0.1-0.3). 3-5 µg S is sufficient to gain accurate and precise results due to the High Sensitivity Method for sulfur, which facilitates a preconcentration of SO_2 sample gas within the Flash EA IRMS unit. However, crucial for achieving this is hardware optimization via the Sulfur Option and the High Performance Sulfur Kit as well as the appropriate maintenance of the system. The system needs to be monitored and SO_2 carry over suppression must be maintained.

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