Multipoint stable isotope correction applied to GC-C-IRMS analysis of endogenous steroids

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September 22nd 2023

IRMS Workshop 2023

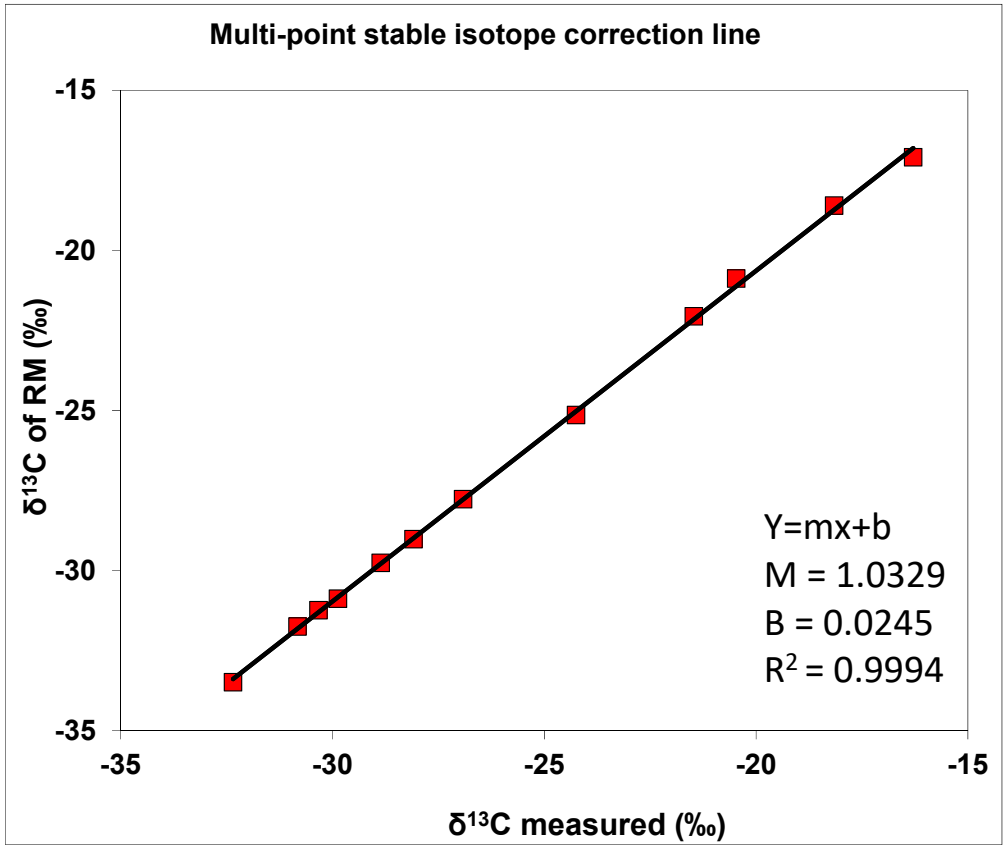
Current Montreal Sample Preparation

Urine Purification via Solid Phase Extraction → Enzymatic Hydrolysis → Liquid-Liquid Extraction → 2-Dimensional HPLC Purification

Isoprime and Isoprime 100
- Modified with additional constant oxygen bleed
  - Increases combustion column efficiency and longevity

Max throughput to date is 120 samples a week

Current Montreal GC-C-IRMS Performs



	Perf-IRMS-A1	Perf-IRMS-A1	Certified $\delta^{13}c$ signature (‰)	Difference Vs Certif. Perform-A
3-Androstanol	**-30.37**	**-31.34**	-31.24	**-0.10**
5β-androstan-3α,17β-diol	**-28.09**	**-28.99**	-29.02	**0.03**
DHEA	**-32.34**	**-33.38**	-33.49	**0.11**
5α-androstan-3α,17β-diol	**-29.87**	**-30.83**	-30.88	**0.05**
Testosterone	**-26.91**	**-27.77**	-27.77	**0.00**
Pregnanediol	**-16.29**	**-16.80**	-17.09	**0.29**
Cholesterol	**-24.25**	**-25.02**	-25.14	**0.12**
16-Androstenol	**-30.82**	**-31.81**	-31.75	**-0.06**
3-Androstanol	**-30.33**	**-31.30**	-31.24	**-0.06**
Étiocholanolone	**-21.47**	**-22.15**	-22.06	**-0.09**
Androsterone	**-20.46**	**-21.11**	-20.88	**-0.23**
Epitestosterone	**-28.86**	**-29.78**	-29.76	**-0.02**
Pregnanediol	**-18.16**	**-18.73**	-18.60	**-0.13**
	Perf-IRMS-B1	Perf-IRMS-B1	Certified $\delta^{13}c$ signature (‰)	Difference Vs Certif. Perform-B

Process of creating our performs

- Step 1 : Finding high purity reference material
  - Impurities in the reference material can lead to larger than expected uncertainties in the certified signatures
- Step 2 : Analysis by EA-IRMS (5 replicates)
  - EA – IRMS analysis is sub contracted to another lab in our case
  - Ensure that there is proper meteorological traceability to the VPDB scale
- Step 3 : Ensure that the $\delta^{13}C$ signatures cover the entire range of expected values
  - Steroids in the middle of the correction curve can be difficult to find
  - This problem is solved by using (for example) existing CRM such as the NMIA MX018 standards
  - In our case we continue with our initial performs due to the longitudinal data this provides us

Verification with externally certified QC (not included in the multipoint calibration)

	QC-MX018-2 Measured	QC-MX018-2 Corrected	Certified $\delta^{13}C$ (‰)	Écart Vs Certif. QC-MX018
5β-Adiol	**-29.61**	**-30.19**	-29.86	**-0.33**
5α-Adiol	**-30.68**	**-31.29**	-31.14	**-0.15**
Épitesto	**-29.80**	**-30.38**	-30.17	**-0.21**
Pd	**-16.39**	**-16.50**	-16.79	**0.29**
11β-HO-A	**-27.96**	**-28.47**	-28.59	**0.12**

Injected at around 5 nA intensity (similar to dosed sample peak heights)
Acceptability criteria of up to 0.4 ‰ difference

MX18-2 (Std 4847)	**Average** $\delta^{13}C$ (‰)	**S.D.** (‰)
5β-Adiol	**-30.04**	**0.12**
5α-Adiol	**-31.06**	**0.12**
Epitestosterone	**-30.33**	**0.13**
Pregnanediol	**-16.67**	**0.14**
11β-Hydroxyandro	**-28.46**	**0.15**

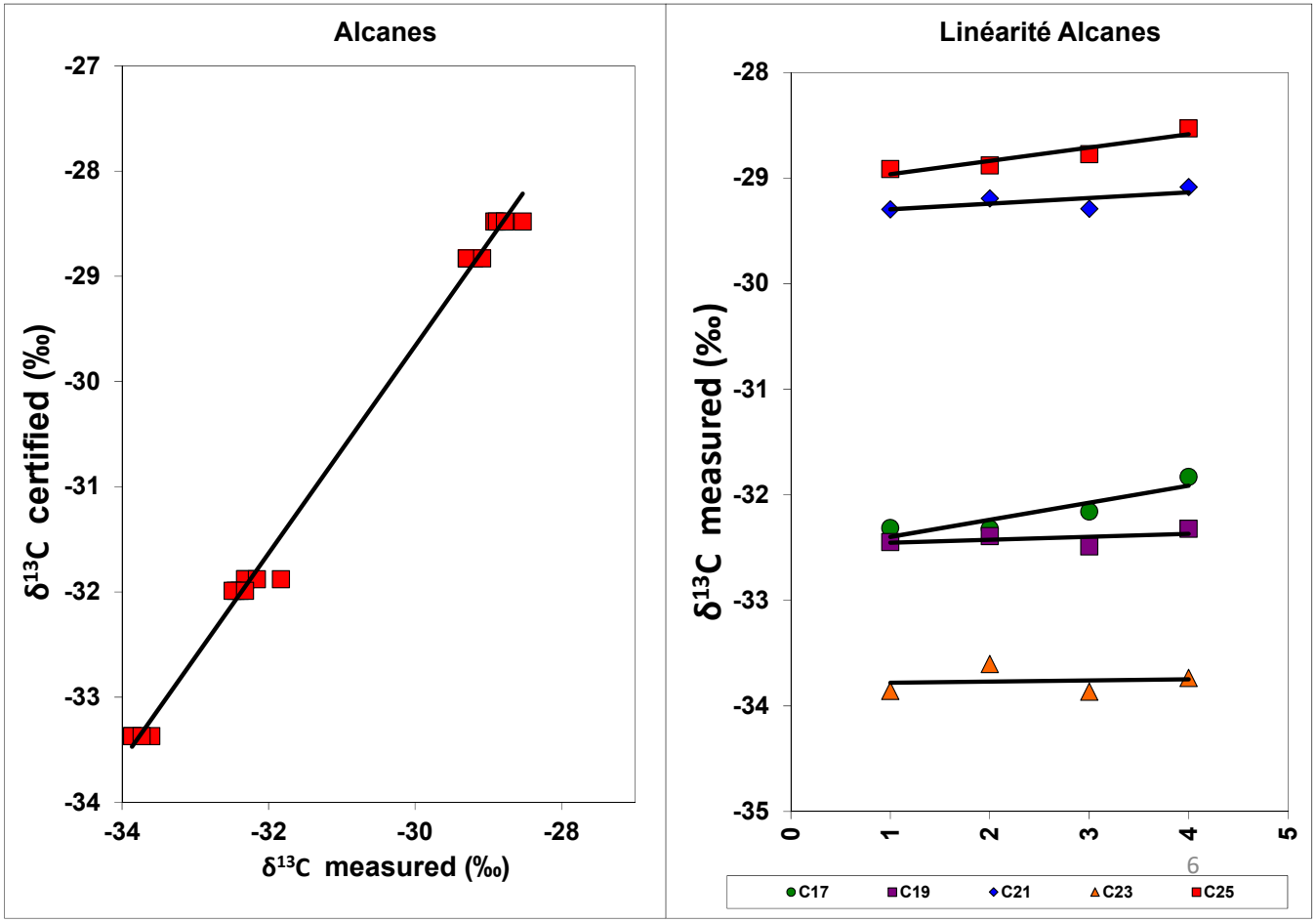
N = 97

Combustion efficiency verified through the comparison with easily combustible RM

Mixture of alcanes from C17 to C25

Serial dilutions in order to demonstrate in-batch linearity

Easily converted to CO_2 relative to steroids
- good marker for improper combustion



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Serial dilutions in order to demonstrate in-batch linearity

Easily converted to CO_2 relative to steroids
- good marker for improper combustion

	Injections				Certified values (‰)
	Alcanes-A	Alcanes-B	Alcanes-C	Alcanes-D	
C17	**-32.48**	**-32.45**	**-32.40**	**-32.24**	-31.88
C19	**-32.67**	**-32.64**	**-32.50**	**-32.65**	-31.99
C21	**-29.25**	**-29.36**	**-29.32**	**-29.66**	-28.83
C23	**-34.06**	**-33.88**	**-34.02**	**-33.99**	-33.37
C25	**-28.91**	**-28.79**	**-28.85**	**-28.93**	-28.48

Alcane	C17	C19	C21	C23	C25
Valeur certifiée	-31.88	-31.99	-28.83	-33.37	-28.48
Moyenne	-32.39	-32.61	-29.40	-33.99	-28.87
Ecart type	**0.11**	**0.07**	**0.18**	**0.08**	**0.06**
Ecart Vs Certif.	**-0.51**	**-0.62**	**-0.57**	**-0.62**	**-0.39**

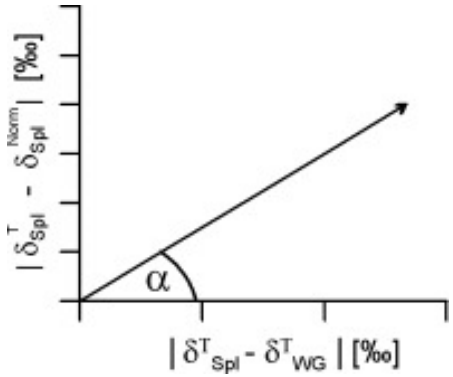
Time to replace or re-oxydize the combustion reactor!

IRMS normalisation/anchoring strategy

- Four principle mechanisms of ensuring traceability to primary standards
  - Single point anchoring using CO_2
  - Single point anchoring using certified reference material
  - Two-point anchoring using 2 certified reference compounds
  - Multi-point anchoring using >2 certified reference compounds

Single point anchoring using CO_2

- Single point anchoring: Certify the ~~reference~~ monitoring gas and use it to determine unknown samples $\delta^{13}C$ signature
  - This method is **no longer accepted in other fields**
  - The "reference" gas is now called a "monitoring or working" gas to prevent confusion
  - Monitoring gases are usually around -40 ‰. The further you are from this $\delta^{13}C$, the larger your error is

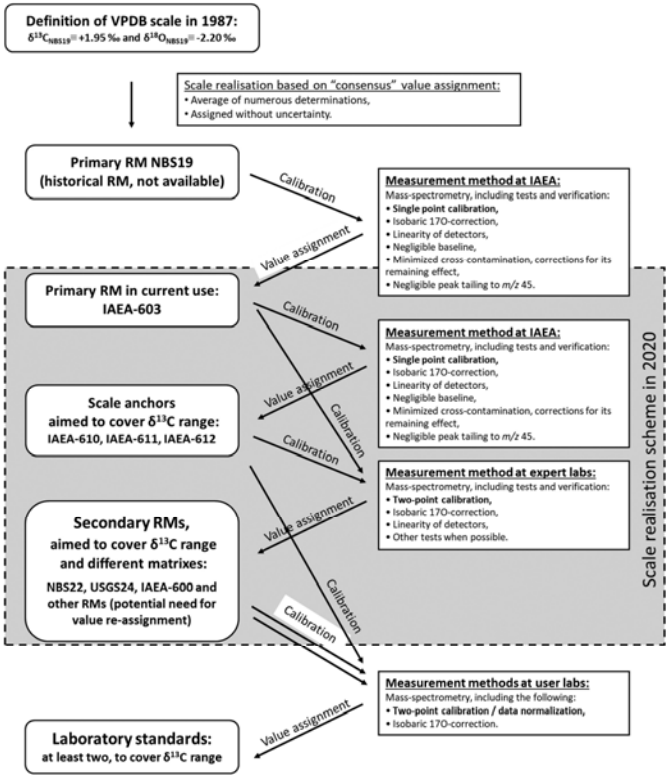


Paul, Skrzypek and Forizs, Rapid Comm. Mass Spec (2007)

Working Gas vs Reference Gas

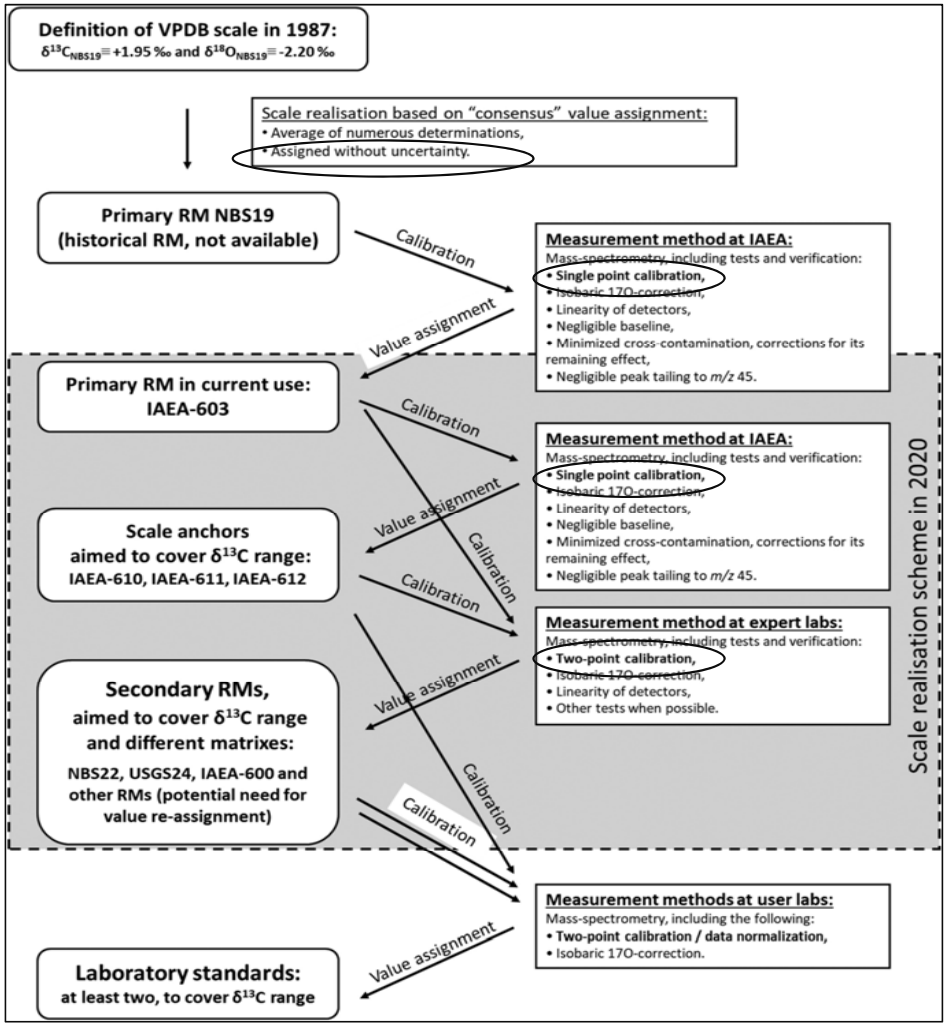
- Stable isotope signature of the working gas is anchored to the VPDB scale using NIST standards
  - Carbonate rocks treated with acid in order to form CO_2
  - Measured using dual inlet IRMS
  - Not perfect – e.g. arguments over the “True” value for NBS-19

- In Canada we have observed a shift in isotope signature of our working gas cylinders from -40 ‰ to -10 ‰ and even -5‰



Rapid Comm Mass Spectrometry, Volume: 35, Issue: 8, First published: 07 December 2020, DOI: (10.1002/rcm.9018)

“True” Values?



Rapid Comm Mass Spectrometry, Volume: 35, Issue: 8, First published: 07 December 2020, DOI: (10.1002/rcm.9018)

Single point using identical treatment principle

- Anchoring of the working gas stable isotope signature as a function of a single known standard – ideally a steroid in our case

- Produces large normalization errors when far from the “known” signature of the standard

$$\delta_{sp}^{T} = \frac{\left(\delta_{sp}^{M} + 1000\right)\left(\delta_{RM}^{T} + 1000\right)}{\left(\delta_{RM}^{M} + 100\,0\right)} - 1000$$

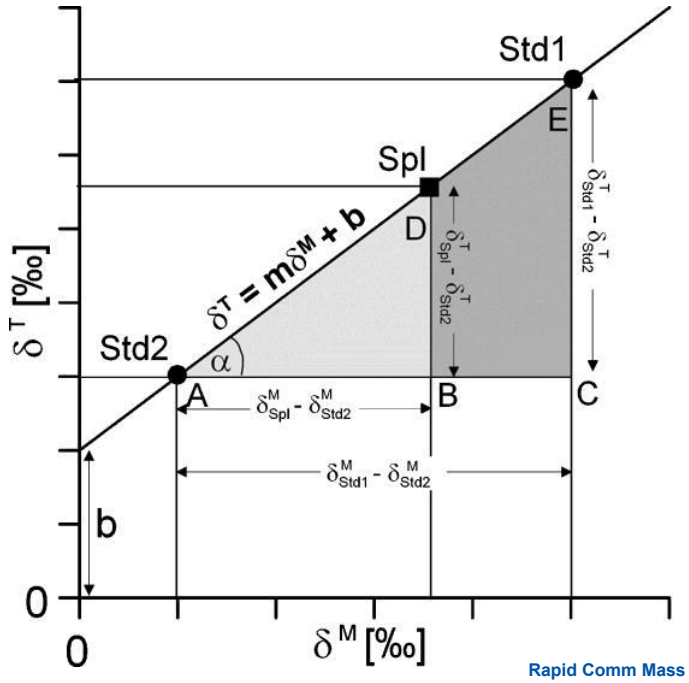
I would not suggest this method for our purposes anyways…
Just because you are using ITP doesn’t mean you are generating good data!

IRMS normalisation/anchoring strategy

- Four principle mechanisms of ensuring traceability to primary standards
  - ~~Single point anchoring using CO_2~~
  - ~~Single point anchoring using certified reference material~~
  - Two-point anchoring using 2 certified reference compounds
  - Multi-point anchoring using >2 certified reference compounds

Two-point anchoring using 2 certified reference compounds

- Analyzing two compounds as CRM and correcting the unknown sample $\delta^{13}C$ signatures



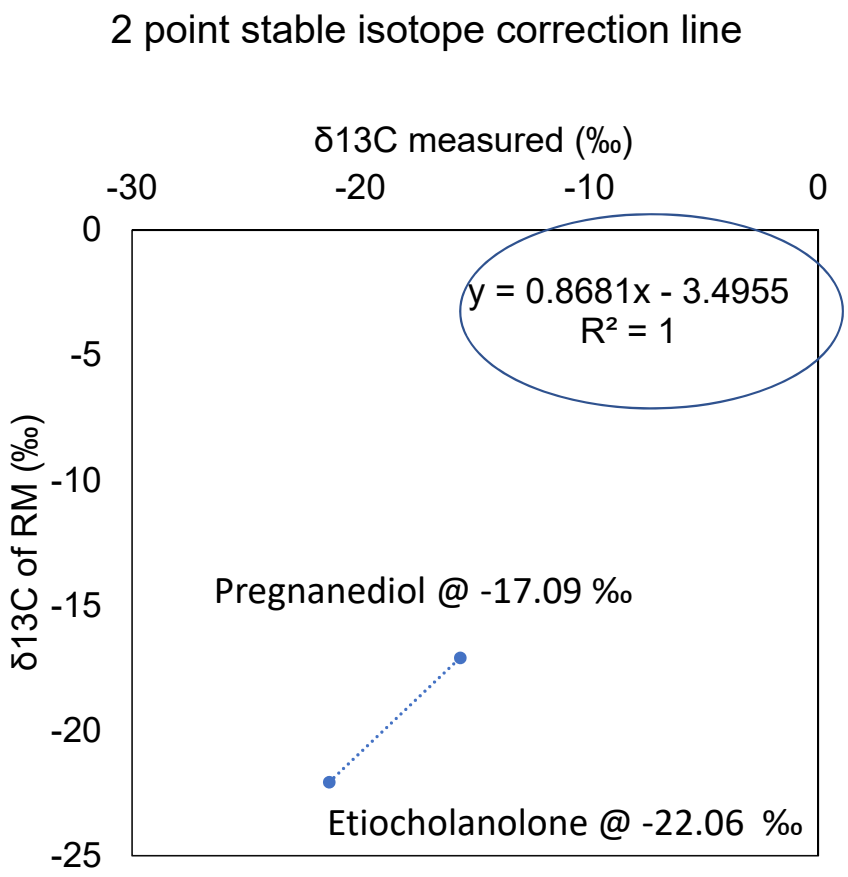
- R squared will always be 1 by definition
  - Not particularly useful information

Tips:

1) Must cover entire range of expected isotope signatures

Rapid Comm Mass Spectrometry, Volume: 21, Issue: 18, Pages: 3006-3014, First published: 17 August 2007, DOI: (10.1002/rcm.3185)

Two-point anchoring using 2 certified reference compounds



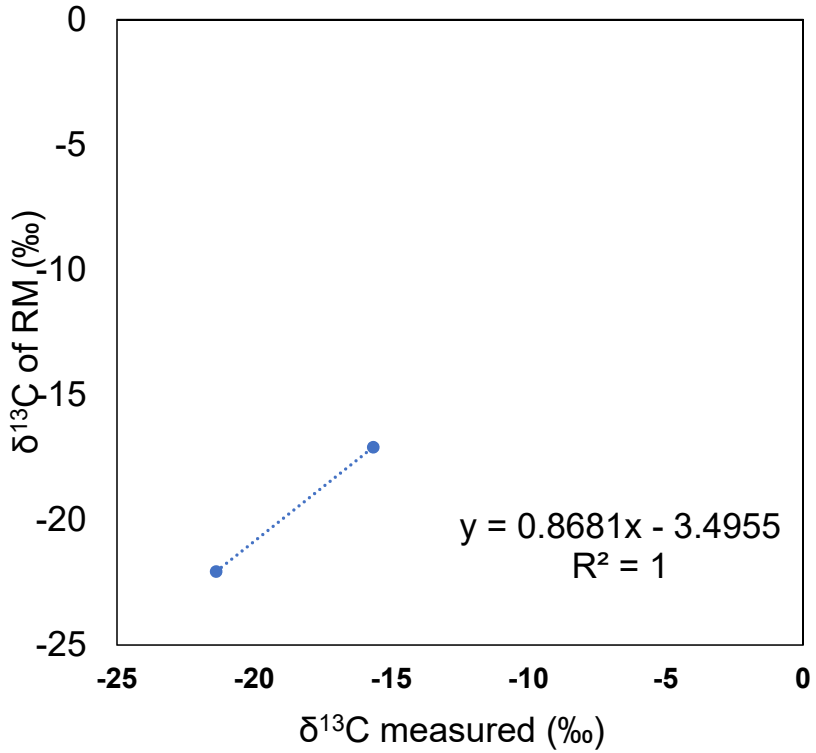
	QC-MX018-2	QC-MX018-2	Valeurs certifiées	Écart Vs Certif. QC-MX018	No. Standard
5β-Adiol	**-28.42**	**-28.17**	-29.86	**1.69**	4847
5α-Adiol	**-29.59**	**-29.18**	-31.14	**1.96**	
Épitesto	**-28.66**	**-28.37**	-30.17	**1.80**	
Pd	**-15.73**	**-17.15**	-16.79	**-0.36**	
11β-HO-A	**-27.22**	**-27.13**	-28.59	**1.46**	

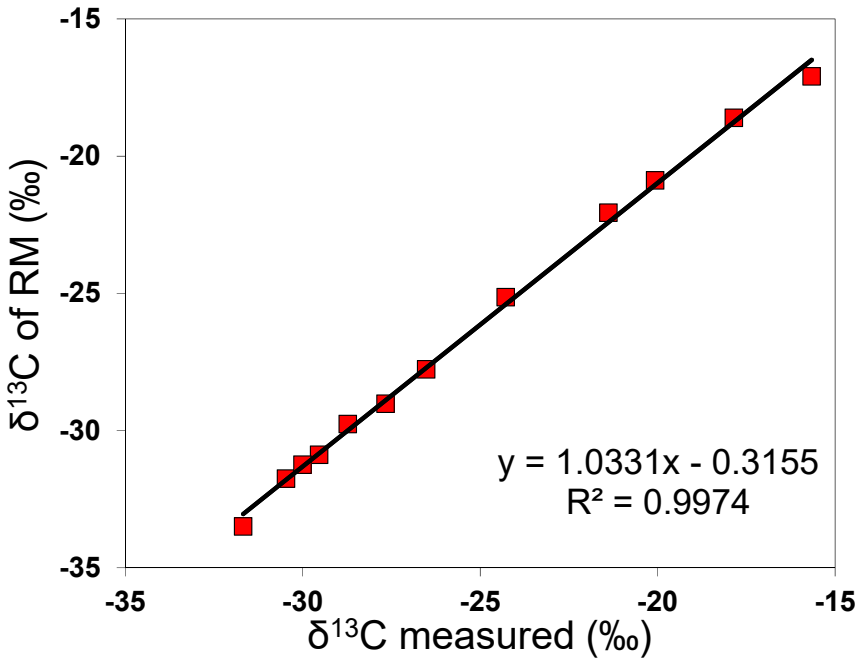
External QC with two calibration

Poorly chosen, does not conver the entire range of expected isotope signatures

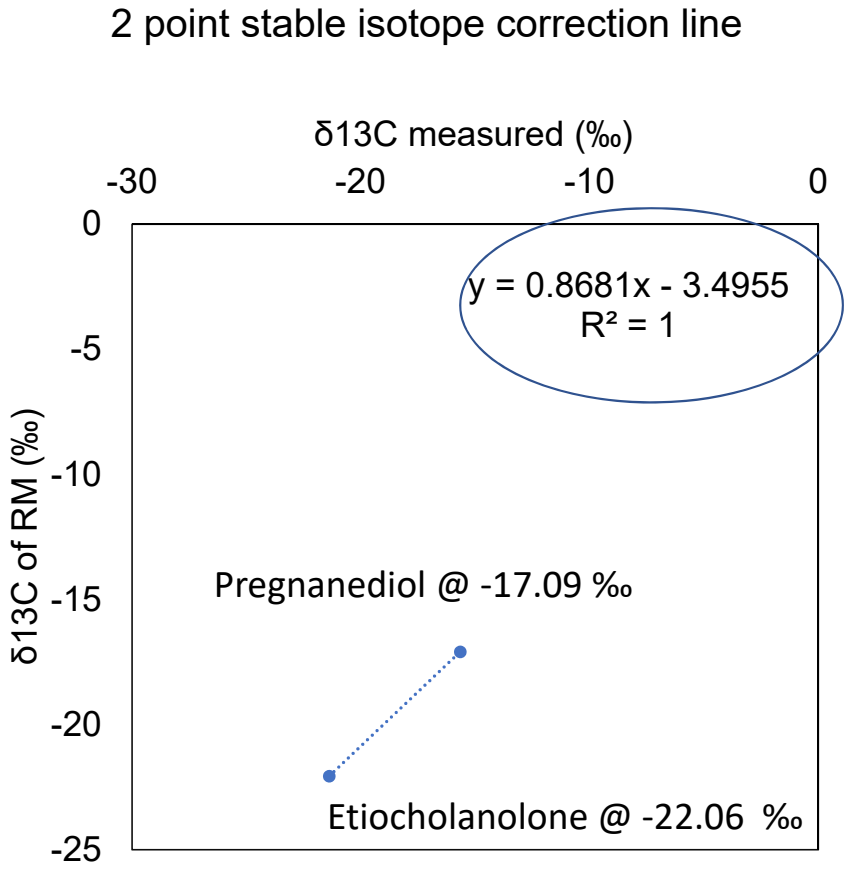
Two point vs multi-point correction

2 point stable isotope correction line





Two point vs multi-point correction



	QC-MX018-2	QC-MX018-2	Certified $\delta^{13}C$ (‰)	Écart Vs Certif. QC-MX018	No. Standard
5β-Adiol	**-28.42**	**-29.68**	-29.86	**0.18**	4847
5α-Adiol	**-29.59**	**-30.88**	-31.14	**0.26**	
Épitesto	**-28.66**	**-29.92**	-30.17	**0.25**	
Pd	**-15.73**	**-16.56**	-16.79	**0.23**	
11β-HO-A	**-27.22**	**-28.44**	-28.59	**0.15**	

External QC with entire multipoint calibration

	QC-MX018-2	QC-MX018-2	Certified $\delta^{13}C$ (‰)	Écart Vs Certif. QC-MX018	No. Standard
5β-Adiol	**-28.42**	**-28.17**	-29.86	**1.69**	4847
5α-Adiol	**-29.59**	**-29.18**	-31.14	**1.96**	
Épitesto	**-28.66**	**-28.37**	-30.17	**1.80**	
Pd	**-15.73**	**-17.15**	-16.79	**-0.36**	
11β-HO-A	**-27.22**	**-27.13**	-28.59	**1.46**	

External QC with two calibration

Poorly chosen, does not conver the entire range of expected isotope signatures

$\delta^{13}C$ outside of the isotopic correction range

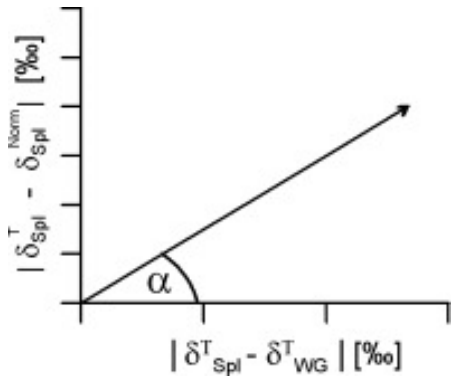
- Isotopically enriched testosterone with an established signature of

-9.5 ‰ consistently measured more enriched than expected (-10.9 ‰)

CO_2 working gas at – 40 ‰

Increased uncertainty the further we are from the CO_2

Mix HPLC	Difference HPLC-Certif.	Valeurs certifiées
-31.5	**-0.22**	-31.2
-34.1	**-0.44**	-33.7
-10.9	**-1.34**	-9.5
-31.2	**0.00**	-31.2
-28.9	**0.09**	-29.0
-30.5	**0.39**	-30.9
-30.2	**-0.40**	-29.8
-16.6	**0.15**	-16.7
-32.2	**-0.45**	-31.8
-31.0	**0.25**	-31.2
-28.8	**0.28**	-29.0
-33.7	**0.30**	-34.0



Altering source parameters corrects the issue
An additional test when altering source parameters

Choice of reference materials to be used

- CO_2
  - Not following ITP
- Indianna university alcane mix
  - Not suitable for steroid calibration
- Create your own
- Use NMIA MX018 (or equivalent)
  - Advantage of being ISO 17034 certified
  - Excellent range in isotope signatures

CERTIFIED REFERENCE MATERIAL
CERTIFICATE OF ANALYSIS

NMIA MX018: Steroid Mixtures certified for Carbon Isotope Delta Value

Certified values

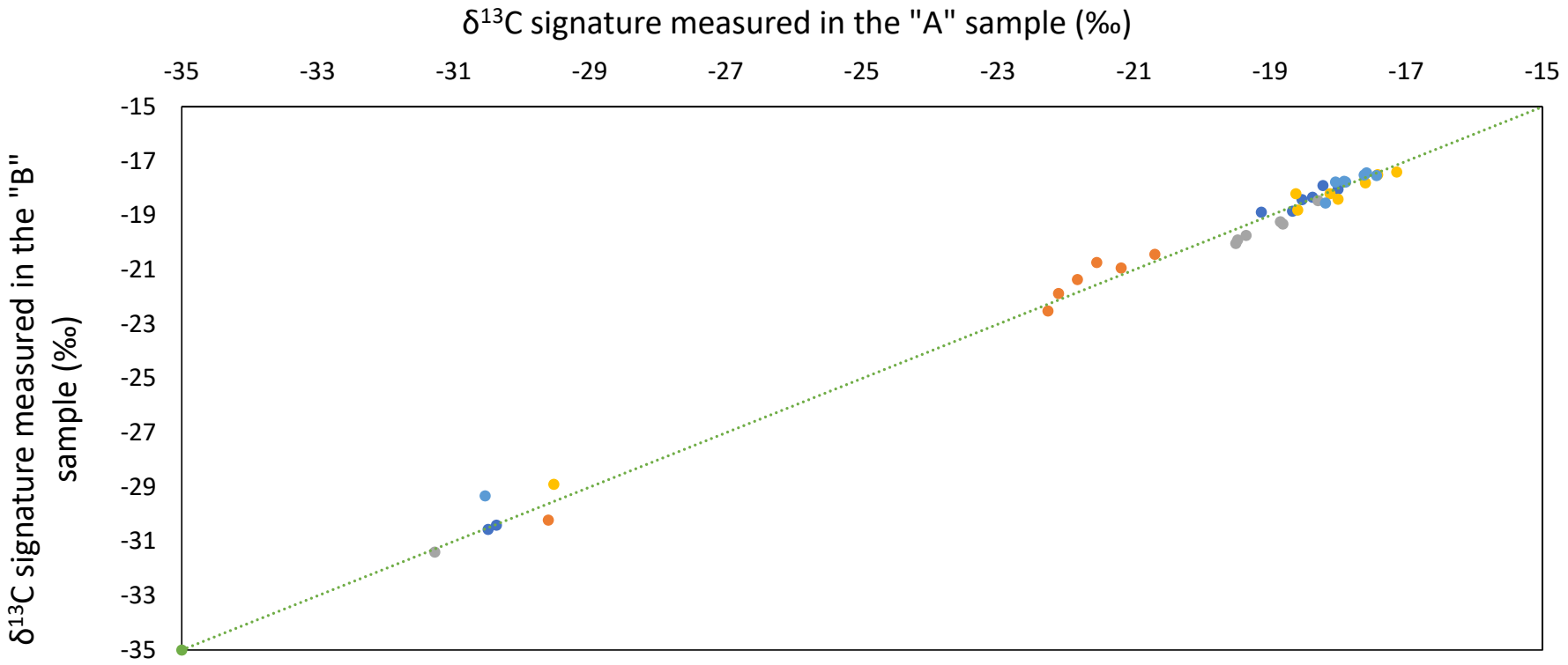
Ampoule		CAS No.	$\delta^{13}C_{VPDB\text{-}LSVEC}$ / ‰	k	V_{off}
MX018-1	Etiocholanolone	53-42-9	–27.94 ± 0.24	2.0	41
Batch No. 2017.12	Androsterone	53-41-8	–27.79 ± 0.21	2.1	15
	11-oxoetiocholanolone	739-27-5	–13.58 ± 0.23	2.1	28
	Testosterone	58-22-0	–27.87 ± 0.24	2.1	24
	11β-hydroxyetiocholanolone	739-26-4	–29.51 ± 0.36	2.0	58
MX018-2	5β-androstane-3α,17β-diol	1851-23-6	–29.86 ± 0.16	2.0	57
Batch No. 2017.12	5α-androstane-3α,17β-diol	1852-53-5	–31.14 ± 0.24	2.0	52
	Pregnanediol	80-92-2	–16.79 ± 0.42	2.0	39
	Epitestosterone	481-30-1	–30.17 ± 0.36	2.0	50
	11β-hydroxyandrosterone	57-61-4	–28.59 ± 0.22	2.0	59
MX018-3	16-androstenol	1153-51-1	–30.96 ± 0.37	2.0	47
Batch No. 2018.01	Dehydroepiandrosterone	53-43-0	–31.63 ± 0.54	2.0	40
	Testosterone	58-22-0	–22.52 ± 0.33	2.0	54

The measurands are the carbon isotope ratio delta values of the stated steroids relative to that embodied in the primary isotopic reference material VPDB on a scale normalised by LSVEC. The uncertainties are expanded to provide a level of confidence of 95%.

Expiry: 30 September 2020

Description: Three ampoules containing dry steroid mixtures in a 2 mL flame sealed ampoule. The ampoules contain approximately 400 µg of each steroid with the exception of 16-androstenol supplied close to 280 µg

Comparing recent A and B sample IRMS results



Perspective from outside of our field

"Neither reviewers nor editors of scientific journals would accept and publish manuscripts reporting quantitative data based on mass spectrometric (MS) analysis if such data were not supported by a multi-point calibration. So, why should manuscripts reporting isotope abundance data based on isotope ratio mass spectrometric(IRMS) analysis be treated any differently?"

Wolfram Meier-
Augenstein & Arndt Schimmelmann (2019) A guide for proper utilisation of stable isotope reference materials*, Isotopes in Environmental and Health Studies, 55:2, 113-
128, DOI: 10.1080/10256016.2018.1538137

Conclusion

A question of robustness

- Use of mutli-point isotope correction line is critical for spotting bad data
- Ought to cover the entire range of expected isotope signatures
- Can be achieved with relatively few additional injections
- Using delta-delta values minimizes these nefarious effect

From a litigation aspect it is imperative that we conform to expected norms