

Quantitative NMR for Carbon and Hydrogen Site-Specific Isotope Analysis: An Emerging Metrological Method

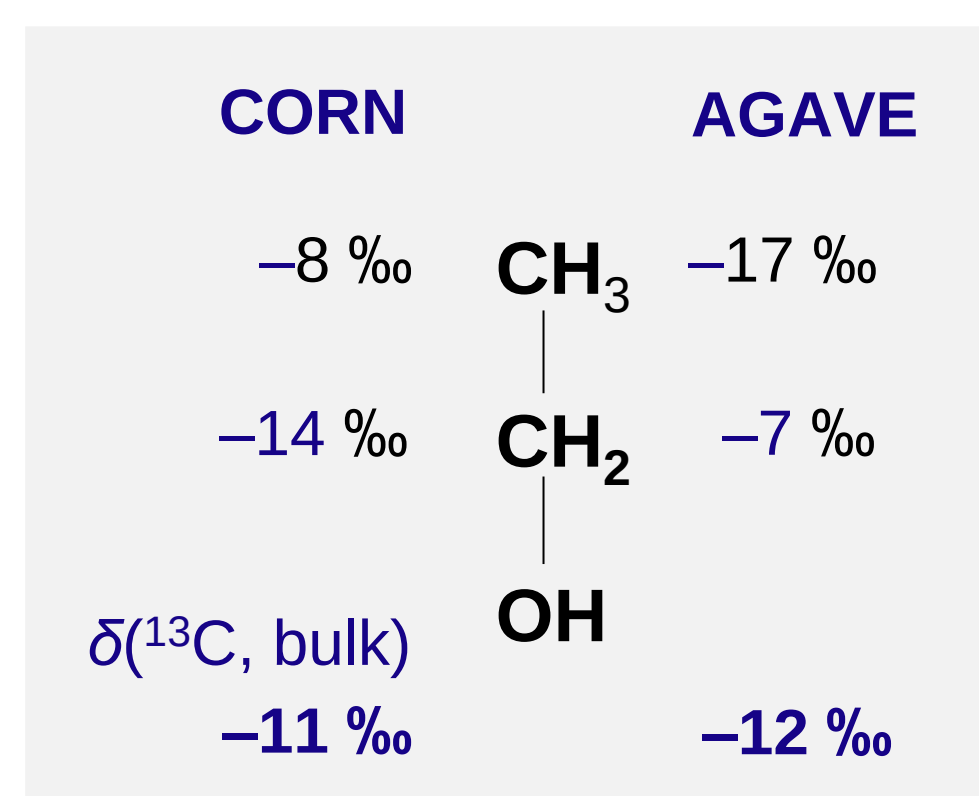
Phuong Mai Le¹, Juris Meija¹, Serge Akoka², Markus Greule³, Stanislav Sokolenko⁴, Andre Simpson⁵, Frank Keppler², Zoltán Mester¹

Metrology, National Research Council Canada, Ottawa, ON, Canada, ²Nantes Université, Nantes, France, ³Heidelberg University, Heidelberg, Germany, ⁴Dalhousie University, Halifax, NS, Canada, ⁵Toronto University, Toronto, ON, Canada

INTRODUCTION

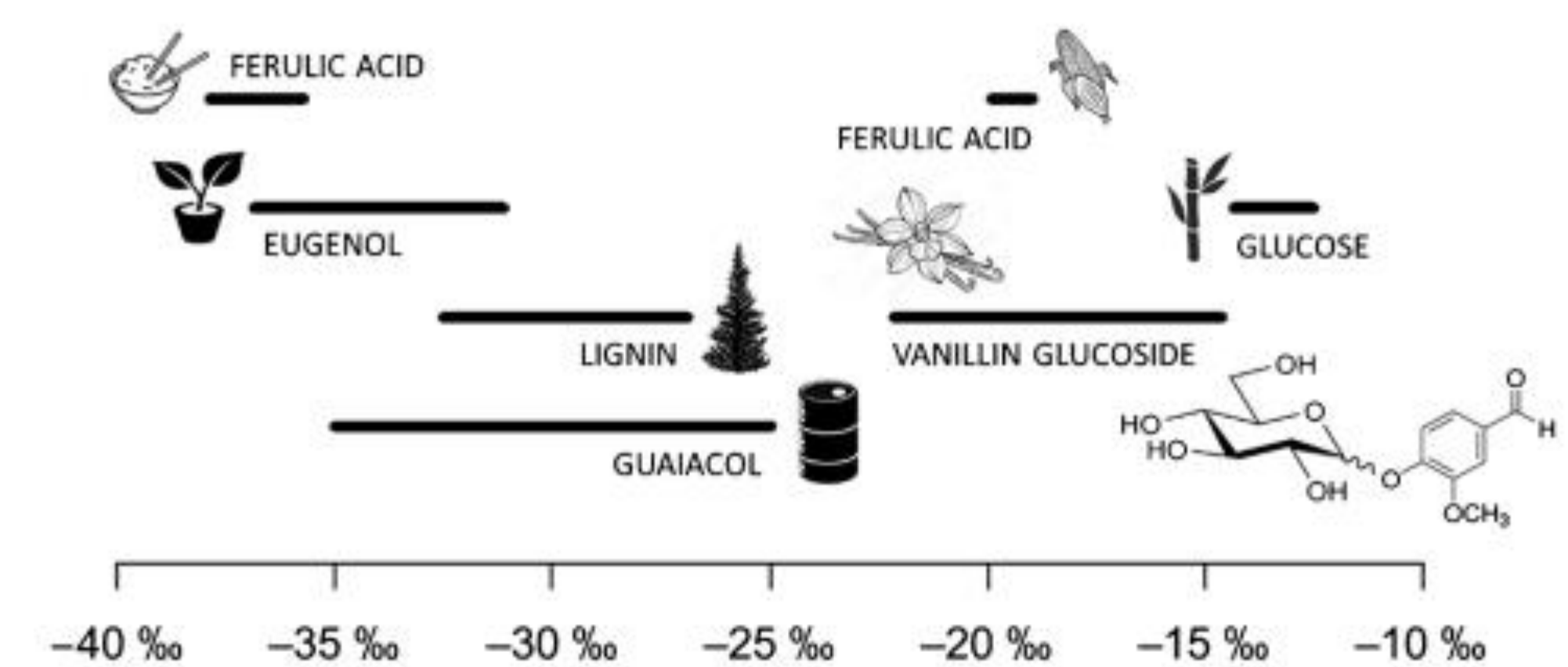
Stable carbon and hydrogen isotope analysis is a powerful technique to identify the provenance of various substances, with applications to metabolite analysis, foods and pharmaceuticals, environmental forensics, and geosciences. Isotope ratio mass spectrometry (IRMS) is commonly used to determine the bulk isotope ratio of the entire molecule. In contrast, quantitative nuclear magnetic resonance (qNMR) allows to determine isotope ratios at individual positions within a molecule (site-specific isotope analysis) without additional chemical modification, which provides additional information for classification of molecules for their geographical, chemical origin and their histories from raw materials to finished products.

Ethanol derived from the corn and agave has similar bulk ¹³C/¹²C isotope ratio. However, their CH₂ and CH₃ carbon isotope site-specific values are significantly different.



Precise measurements of isotopic composition using qNMR can be very challenging for natural substances due to a variety of factors, including the sensitivity. Here, we present rigorous workflows of ¹³C and ²H qNMR methods to provide reliable site-specific measurements of carbon and hydrogen isotope ratios and we validate the results obtained by the qNMR with an independent GC-IRMS method using vanillin samples.

Vanillin, one of the world's most popular flavor used in the food and pharmaceutical industries, is extracted from vanilla beans or obtained (bio)-synthetically. High demand for vanillin and significantly higher costs of natural vanillin than its synthetic alternative leads to counterfeit vanillin. Whereas the traditional IRMS bulk isotope measurements can distinguish between the synthetic and natural vanillin, intramolecular isotopic composition analysis provides significantly more information to better aid the provenance studies.



Natural variations of bulk carbon isotope ratios (isotope delta vs VPDB) obtained by isotope ratio mass spectrometry (IRMS)

MATERIALS

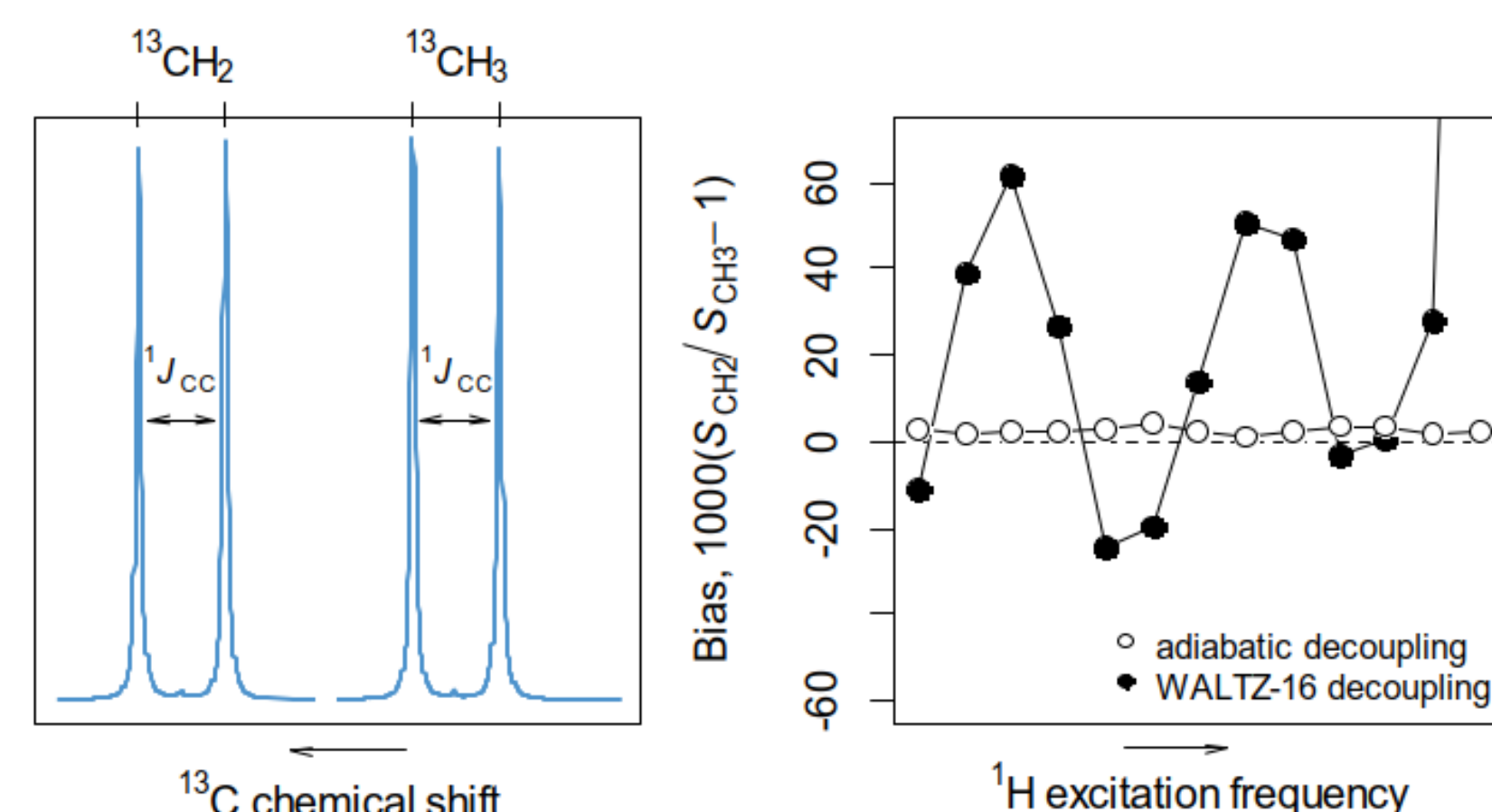
Seven commercial vanillin materials were obtained from difference sources. The chemical purity estimates of each vanillin, expressed in g/g, are 0.9962 ± 0.024 (VANA-1), 0.9954 ± 0.023 (VANB-1), 0.9856 ± 0.039 (VAN-1), 0.9931 ± 0.032 (VAN-4), 0.9932 ± 0.037 (VAN-8), 0.9898 ± 0.030 (VAN-HR), and 0.9695 ± 0.033 (VAN-K).

WORKFLOW FOR ISOTOPIC qNMR

¹³C-qNMR for high-precision, site-specific ¹³C isotope analysis

A high precision (1-2 ‰) is required to analyse ¹³C profile of natural substances. Key considerations to achieve this include:

- The uniformity of ¹H decoupling over the whole ¹H chemical range
- Use of ¹³C-NMR with adiabatic decoupling pulse
- NMR spectrometer requires a performance evaluation using doubly-labelled ethanol as a molecule probe for sensitivity and accuracy tests



Spectrometer qualification using adiabatic and standard waltz decoupling pulses on double-labelled ¹³C₂-ethanol

- NMR parameters should be meticulously optimized which include the elimination of nOe and partial saturation, use of reasonable acquisition times, use of ¹³C-zigzag pulse with 90° flip angle, large inter-pulse delay, and relaxation reagent.

²H-qNMR for high-precision, site-specific ²H isotope analysis

Key considerations to achieve good measurements include

- NMR spectrometer equipped a 'deuterium' NMR probe, stabilization (lock) channel at ¹⁹F frequency and ¹H pulse decoupling applied during ²H-qNMR acquisition
- NMR spectrometer evaluation using three ethanol standards and TMU reference material with certified ²H/¹H ratios

Site-specific ²H/¹H values in ppm and their standard uncertainties from three ERM ethanol reference materials, measured by ²H-qNMR

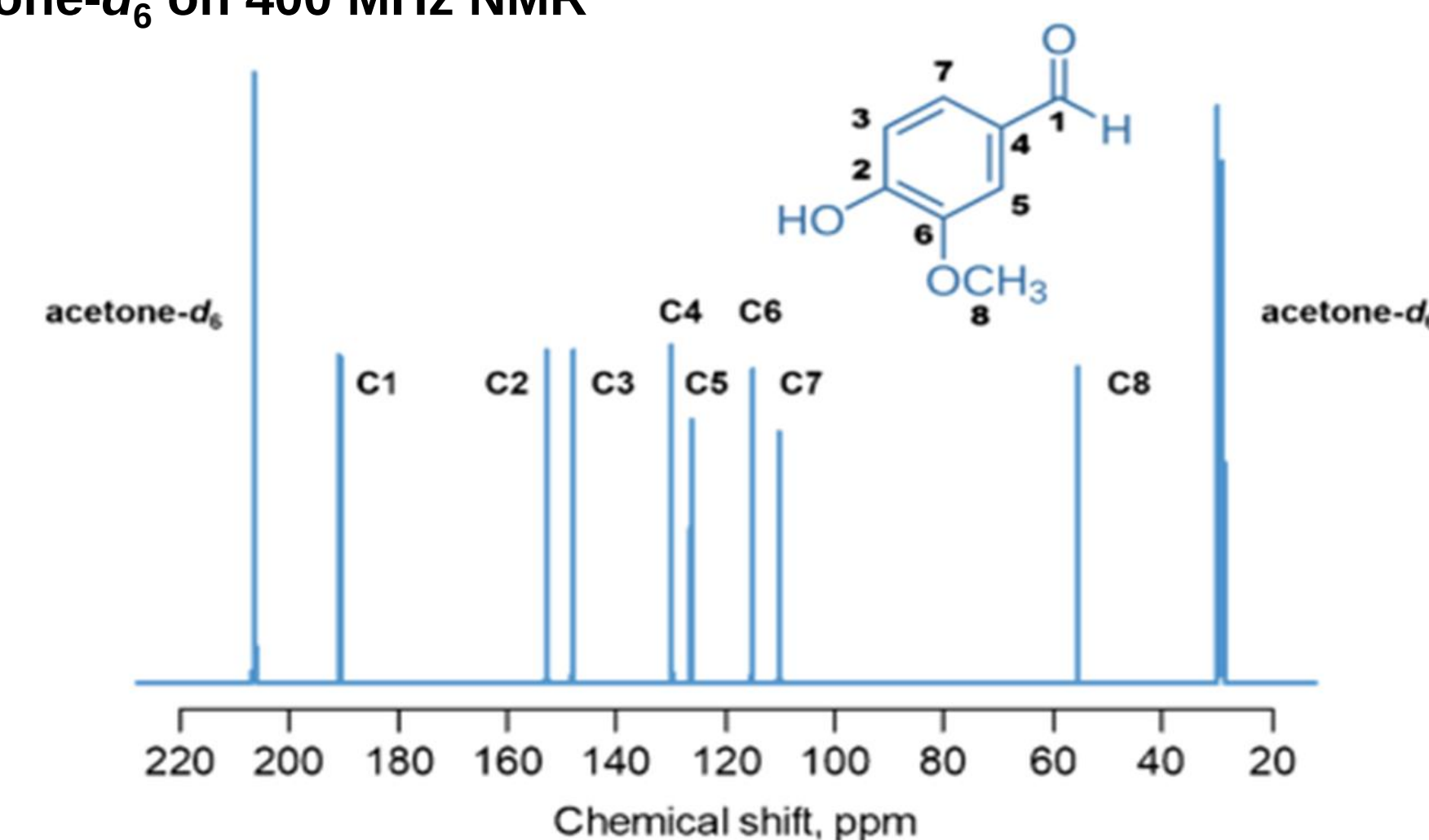
CRM		$R(^2H/^1H, CH_2)$	$R(^2H/^1H, CH_3)$
		ppm	ppm
EtOH-H	NRC	129.9 ± 0.5	111.6 ± 0.7
	ERM certified	129.9 ± 0.2	110.5 ± 0.1
EtOH-M	NRC	130.1 ± 0.5	104.3 ± 0.7
	ERM certified	130.5 ± 0.1	103.3 ± 0.1
EtOH-L	NRC	125.5 ± 0.5	93.4 ± 0.7
	ERM certified	125.6 ± 0.4	92.6 ± 0.1

Data Processing and treatment of qNMR signals

In order to achieve high-precision results, robust peak area evaluation is required. To achieve this, a new deconvolution (NMRFIT) algorithm was developed using both the real and imaginary components of the NMR data to fit NMR data peaks^[1].

RESULTS

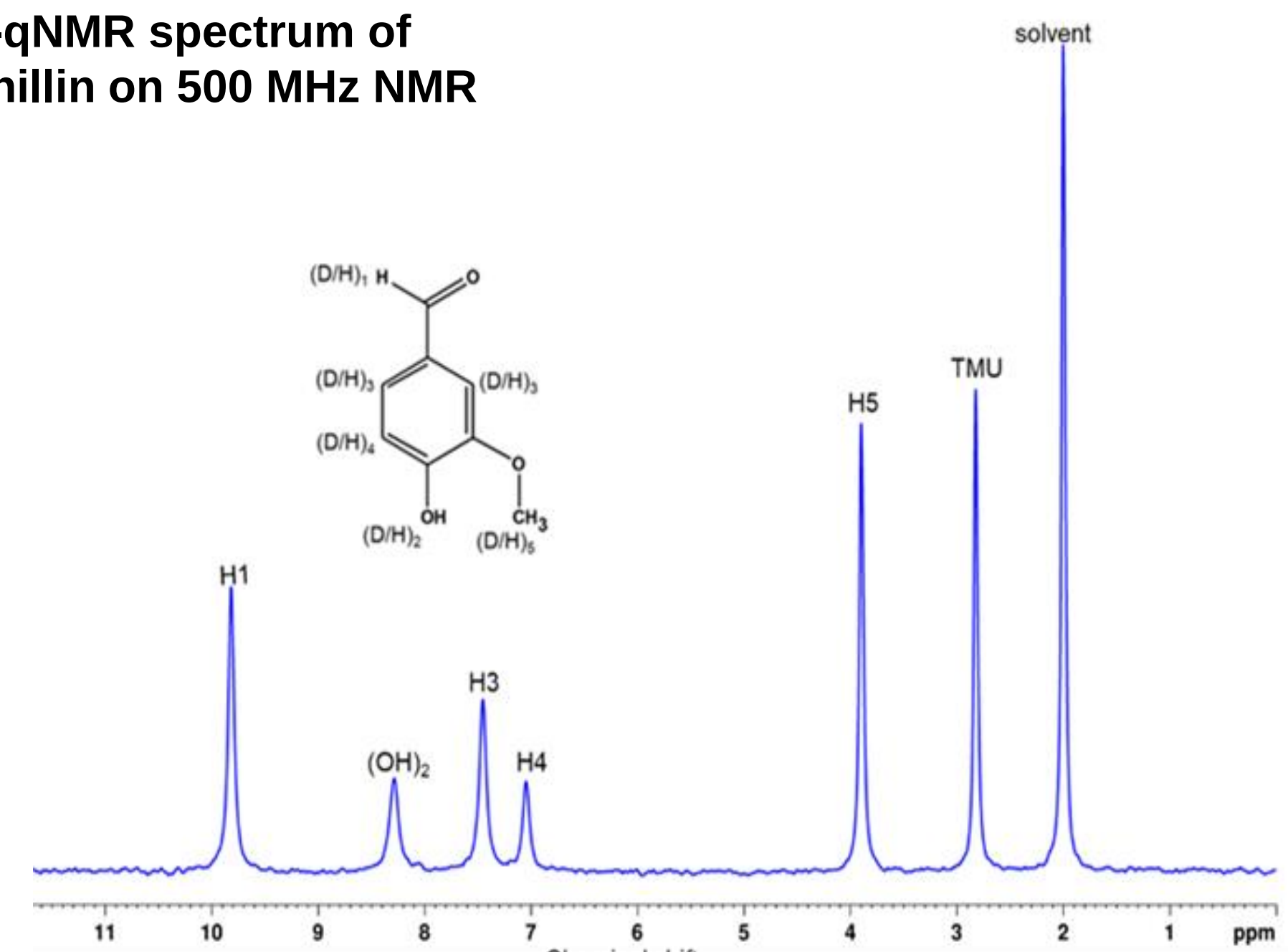
¹³C-NMR spectrum of vanillin in acetone-d₆ on 400 MHz NMR



Site-specific carbon isotope delta values in select vanillin samples measured by ¹³C-qNMR. Values reported in permilles vs VPDB [2-4]

Site	VANA-1	VANB-1	VAN-1	VAN-4
bulk	-31.30 ± 0.06	-25.85 ± 0.05	-29.81 ± 0.06	-31.10 ± 0.08
C1	-21.36 ± 0.7	-20.07 ± 0.7	-22.81 ± 0.7	-19.98 ± 0.7
C2	-32.09 ± 0.7	-29.87 ± 0.7	-32.94 ± 0.7	-28.88 ± 0.7
C3	-32.52 ± 0.7	-32.77 ± 0.7	-33.24 ± 0.7	-31.28 ± 0.7
C4	-28.26 ± 0.7	-24.48 ± 0.7	-31.64 ± 0.7	-26.83 ± 0.7
C5	-29.84 ± 0.7	-23.85 ± 0.7	-23.31 ± 0.7	-28.08 ± 0.7
C6	-29.38 ± 0.7	-26.38 ± 0.7	-27.53 ± 0.7	-26.80 ± 0.7
C7	-23.73 ± 0.7	-18.82 ± 0.7	-21.04 ± 0.7	-24.59 ± 0.7
C8	-53.25 ± 0.7	-30.59 ± 0.7	-46.53 ± 0.7	-62.36 ± 0.7

²H-qNMR spectrum of vanillin on 500 MHz NMR

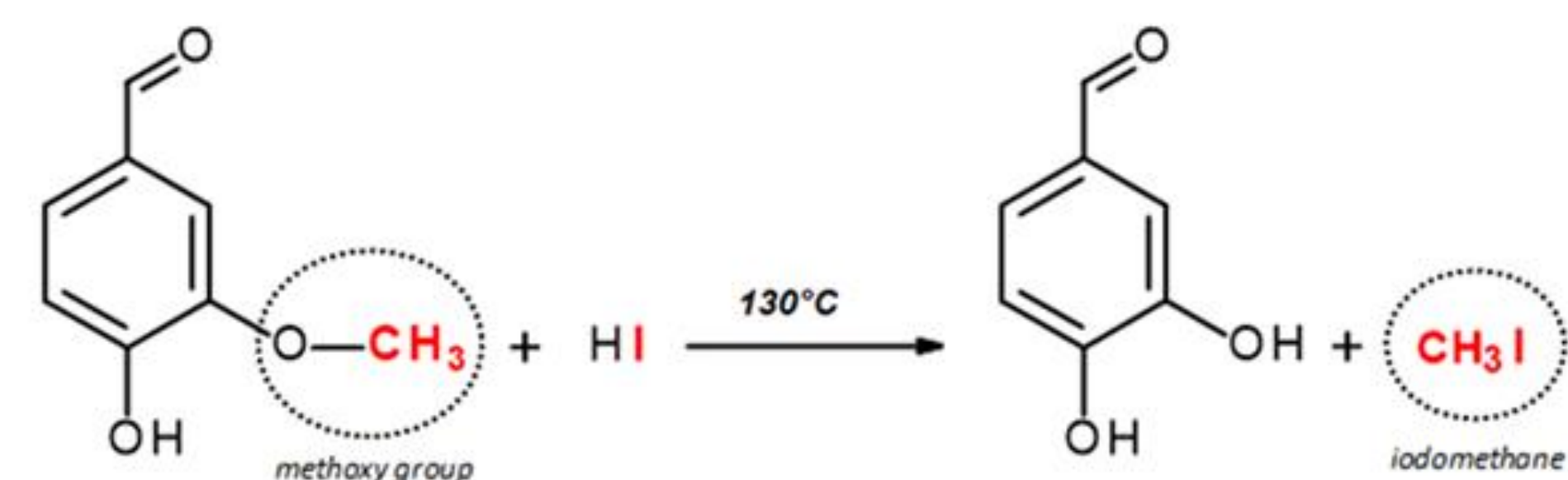


Site-specific hydrogen isotope ratios for select vanillin samples measured by ²H-qNMR. Values reported in ppm^[5]

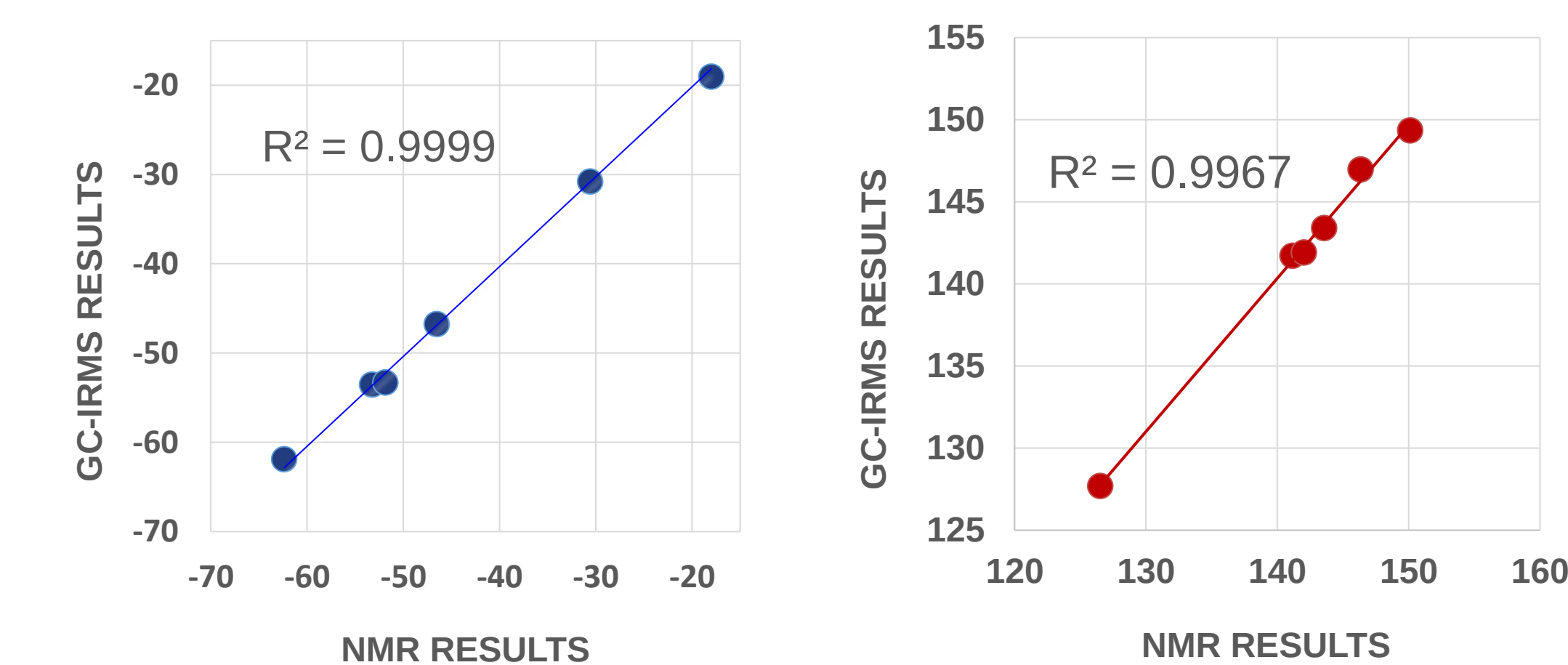
Material	$R(^2H/^1H, H1)$	$R(^2H/^1H, H3)$	$R(^2H/^1H, H4)$	$R(^2H/^1H, H5)$
VANA-1	330.0 ± 2.2	138.9 ± 1.4	142.4 ± 3.6	143.6 ± 0.9
VANB-1	267.8 ± 2.2	139.4 ± 1.4	147.1 ± 3.6	146.3 ± 0.9
VAN-1	258.5 ± 2.2	133.1 ± 1.4	140.9 ± 3.6	141.2 ± 0.9
VAN-4	305.3 ± 2.2	142.8 ± 1.4	146.8 ± 3.6	142.0 ± 0.9

VALIDATION

Results of ¹³C-qNMR and ²H-qNMR methods for vanillin were validated using an independent measurement strategy that relies on GC-IRMS and chemical cleavage of the vanillin methoxy group.



- GC-IRMS measurements provide significantly smaller measurement uncertainties for carbon and hydrogen isotopes of vanillin methoxy group, while also requiring considerably smaller sample size compared to qNMR. Remarkably consistent results from six vanillin samples were obtained by two these methods.
- qNMR affords isotopic measurements of all carbon and hydrogen atoms (except for OH) of the vanillin while the GC-IRMS measures only those of the methoxy group.



Interlaboratory comparison of qNMR and GC-IRMS methods for the isotopic composition of vanillin methoxy group (C8 and H5).

(left) ¹³C/¹²C isotope deltas, reported in permilles vs VPDB. (right) ²H/¹H isotope ratios, reported in ppm

CONCLUSIONS

- qNMR and GC-IRMS can be considered as complementary analytical methods, and their combined use provides reliable results. The variability of site-specific isotope ratios provides further ability to discern synthetic histories of vanillin.
- The reliability of qNMR site-specific isotope measurements supported the production of carbon isotopic certified reference materials of vanillin, VANA-1 and VANB-1 at the NRC.
- The isotope analysis method has been extended to analyze carbon and hydrogen isotope composition of methyl *tert*-butyl ether (MTBE), a common industrial groundwater contaminant.
- Our poster highlights the innovation and application of NMR creating an emerging metrological qNMR method for authentication of compounds and studies of their metabolism in different areas.

REFERENCES

- Sokolenko S et al., *J. Meg. Res.* **2019**, 298, 91-100.
- Le PM et al. *Anal. Bioanal. Chem.* **2022**, 414, 7153-7165
- Chartrand M et al. *Anal. Bioanal. Chem.* **2022**, 414, 7877-7883
- Greule M et al., *JASMS* **2024**, 35,100-105.
- Le PM et al. Deuterium isotope ratio measurement using ²H-qNMR and GC-IRMS: manuscript in preparation **2025**