Certification of nitrate in SPIN-1 spinach powder reference material by high-precision isotope dilution GC–MS (ESM)

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S1 Certification: GC–MS instruments and settings at the NRC lab

In this paragraph, instruments and settings used at the National Research Council Canada for the SPIN-1 certification are presented.

S1.1 GC–MS in EI mode

An Agilent 7000 TripleQuad MS equipped with an Agilent 7890A GC and with a CTC PAL3 autosampler was used for EI acquisitions. 10 mL volume headspace vials were employed for the analysis. The vials were incubated without agitation for 12 s at 35 °C and 100 µL of headspace were sampled and injected in the GC (10:1 split ratio at 120 °C, liner: 1 mm ID × 6.3 mm OD × 78.5 mm length). The headspace syringe was kept at 70 °C and flushed for 3 min with N_2 after every injection. The chromatography was recored within 3 min on the isotherm at 30 °C with a constant He flow of 1.2 mL/min. A (5%-phenyl)-methylpolysiloxane DB-5.625 column was used for separation: 30 m length × 0.250 mm ID × 0.25 µm film. The transfer line was set at 220 °C. The $EtONO_2$ derivative was detect at 2.6 min by mass spectrometry in EI SIM mode on the signals:

$^{14}NO_2^+$, m/z 46
$^{15}NO_2^+$, m/z 47
$CH_2O^{14}NO_2^+$, m/z 76
$CH_2O^{15}NO_2^+$, m/z 77

Each signal was acquired with a 50 ms dwell time. The ion source was kept at 150 °C and the EI ionization was obtained at 70 eV. The quadrupoles were kept at 150 °C and no voltage was applied to the collision cell (quench gas: He 2.25 mL min^{-1}; collision gas: N_2 1.5 mL min^{-1}). The gain factor was set to 1 and all the other setting were defined by standard MS autotune. The same instrument was employed for acquisition in EI MRM mode. 10 mL volume headspace vials were employed for the analysis. The vials were incubated without agitation for 12 s at 35 °C and 1 mL of headspace were sampled and injected in the GC (100:1 split ratio at 120 °C, liner: 1 mm ID × 6.3 mm OD × 78.5 mm length). The headspace syringe was kept at 70 °C and flushed for 3 min with N_2 after every injection. The chromatography was recored within 3 min on the isotherm at 30 °C with a constant He flow of 1.2 mL/min. A (5%-phenyl)-methylpolysiloxane DB-5.625 column was used for separation: 30 m length × 0.250 mm ID × 0.25 µm film. The transfer line was set at 220 °C. The $EtONO_2$ derivative was detect at 2.6 min by mass spectrometry in EI MRM mode on the transitions:

$$CH_2O^{14}NO_2{}^+ \rightarrow {}^{14}NO_2{}^+,\ m/z\ 76 \rightarrow 46$$
$$CH_2O^{15}NO_2{}^+ \rightarrow {}^{15}NO_2{}^+,\ m/z\ 77 \rightarrow 47$$

Each transition was acquired with a 50 ms dwell time. The ion source was kept at 150 °C and the EI ionization was obtained at 70 eV. The quadrupoles were kept at 150 °C and 10 V was applied to the collision cell (quench gas: He 2.25 mL min^{-1}; collision gas: N_2 1.5 mL min^{-1}). The gain factor was set to 1 and all the other setting were defined by standard MS autotune.

S1.2 GC–MS in CI mode

A Hewlett-Packard 5973 MS equipped with a 6890A GC and a CTC CombiPAL autosampler was used for PCI SIM acquisitions. 10 mL volume headspace vials were employed for the analysis. The vials were incubated without agitation for 30 s at 35 °C and 100 µL of headspace were sampled and injected in the GC (10:1 split ratio at 120 °C, liner: 1 mm ID × 6.3 mm OD × 78.5 mm length). The headspace syringe was kept at 70 °C and flushed for 145 s with N_2 after every injection. The chromatography was recored within 3 min on the isotherm at 30 °C with a constant He flow of 1.2 mL/min. A (5%-phenyl)-methylpolysiloxane DB-5.625 column was used for separation: 30 m length × 0.250 mm ID × 0.25 µm film. The transfer line was set at 220 °C. The $EtONO_2$ derivative was detect at 2.5 min by mass spectrometry in PCI SIM mode on the signals:

$$CH_3CH_2O^{14}NO_2{-}H^+,\ m/z\ 92$$
$$CH_3CH_2O^{15}NO_2{-}H^+,\ m/z\ 93$$

Each signal was acquired with a 50 ms dwell time. The ion source was kept at 250 °C and the CI ionization was obtain with CH_4 using a standard autotune routine for MS calibration. The same instrument was employed for NCI SIM acquisitions. 10 mL volume headspace vials were employed for the analysis. The vials were incubated without agitation for 30 s at 35 °C and 100 µL of headspace were sampled and injected in the GC (100:1 split ratio at 120 °C, liner: 1 mm ID × 6.3 mm OD × 78.5 mm length). The headspace syringe was kept at 70 °C and flushed for 145 s with N_2 after every injection. The chromatography was recored within 3 min on the isotherm at 30 °C with a constant He flow of 1.2 mL/min. A (5%-phenyl)-methylpolysiloxane DB-5.625 column was used for separation: 30 m length × 0.250 mm ID × 0.25 µm film. The transfer line was set at 220 °C. The $EtONO_2$ derivative was detect at 2.5 min by mass spectrometry in NCI SIM mode on the signals:

$$^{14}NO_2^-,\ m/z\ 46$$
$$^{15}NO_2^-,\ m/z\ 47$$

Each signal was acquired with a 50 ms dwell time. The ion source was kept at 150 °C and the CI ionization was obtain with CH_4 using a standard autotune routine for MS calibration.

S1.3 Integration of GC–MS chromatograms

The integration of all chromatograms was obtained with the Agilent MassHunter software for quantitative analysis with the Agile integrator. The chromatographic peak areas were used for the estimation of the isotope ratios r_{AB}. For example, in NCI SIM mode the area of the $EtONO_2$ peak extracted at m/z 46 (A_A) was divided by the area of the $EtONO_2$ peak extracted at m/z 47 A_B:

$$r_{AB} = \frac{A_A}{A_B} \tag{1}$$

The r_{AB} were than used for isotope dilution calculation.

S2 Validation of the IDMS method: ESM

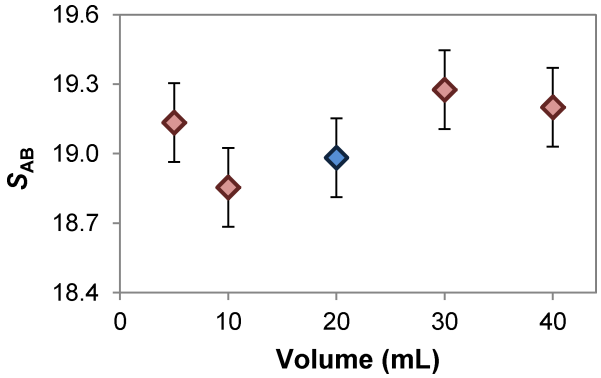

Figure S1: 0.2 g of SPIN-1 were extracted with different water volumes at 70 °C for 15 min). No difference in the response was observed

Table S1: Isotope ratios measured in the natural materials in EI mode: SPIN-1 and primary standards[a]

Injection	EI SIM 46/47			EI SIM 76/77			EI MRM		
	$r_{A^*\text{-}1}$	$r_{A^*\text{-}2}$	r_A	$r_{A^*\text{-}1}$	$r_{A^*\text{-}2}$	r_A	$r_{A^*\text{-}1}$	$r_{A^*\text{-}2}$	r_A
1	220.6	205.6	210.6	62.98	61.22	61.13	230.9	225.9	222.5
2	215.7	205.6	208.4	64.09	65.88	63.84	238.2	242.9	224.8
3	207.2	208.8	210.0	63.16	59.11	63.83	234.1	239.7	224.7
4	213.9	217.9	191.2	66.89	63.05	62.83	227.2	234.2	250.2
5	204.1	213.7	202.0	64.71	64.01	64.93	235.0	233.1	230 .0

[a] $r_{A^*\text{-}1}$ isotope ratio measured in the NIST SRM 3185 Lot.No. 050517
$r_{A^*\text{-}2}$ isotope ratio measured in the Sigma-Aldrich Prod.No. 74246 Lot.No. BCBT3311
r_A isotope ratio measured in the NRC SPIN-1 CRM.

Table S2: Isotope ratios measured in the natural materials in CI mode: SPIN-1 and primary standards[a]

Injection	PCI SIM			NCI SIM		
	$r_{A^*\text{-}1}$	$r_{A^*\text{-}2}$	r_A	$r_{A^*\text{-}1}$	$r_{A^*\text{-}2}$	r_A
1	37.97	36.48	30.34	106.2	104.8	102.9
2	34.91	36.69	33.84	100.0	100.6	99.0
3	32.84	31.14	37.13	97.6	97.8	95.8
4	36.52	30.38	32.34	98.2	96.2	95.8
5	32.30	37.13	32.38	94.8	94.7	93.8

[a] same legend as Table S1.

Table S3: Ratios between nitrate data generated by different MS methods (n = 114). Standard deviations are reported in parenthesis

Comparing[a]	**Ratio (SD)**
EI SIM 46/47 *vs* EI SIM 76/77	1.0021 (0.0053)
EI SIM 46/47 *vs* EI MRM	1.0003 (0.0047)
EI SIM 46/47 *vs* PCI SIM	1.0010 (0.0056)
EI SIM 46/47 *vs* NCI SIM	1.0005 (0.0038)
EI SIM 76/77 *vs* EI MRM	0.9982 (0.0050)
EI SIM 76/77 *vs* PCI SIM	0.9989 (0.0079)
EI SIM 76/77 *vs* NCI SIM	0.9984 (0.0034)
EI MRM *vs* PCI SIM	1.0008 (0.0073)
EI MRM *vs* NCI SIM	1.0002 (0.0040)
PCI SIM *vs* NCI SIM	0.9995 (0.0063)

[a] The EI-MS detection was performed in SIM (isotope ratios: 46/47 and 76/77) and MRM (isotope ratio: 76$\rightarrow$46/77$\rightarrow$47) with the Agilent 7000 Triple-Quad. The SIM CI-MS detection was performed in positive (isotope ratio: 92/93) and negative (isotope ratio: 46/47) mode with the HP 5973 GCMS system

S3 Production and certification of the SPIN-1: ESM

Table S4: Quadruple isotope dilution GC–MS: relative contribution of the major uncertainty components (n = 114)[a]

Detection mode	r_{AB}	$r_{A^*B\text{-}2}$	$w_{A^*\text{-}2}$	m_A
EI SIM 46/47	29%	37%	28%	4%
EI SIM 76/77	36%	36%	24%	2%
EI MRM	42%	22%	30%	4%
PCI	40%	49%	10%	1%
NCI	22%	22%	45%	6%

[a] The variance contribution of all other variables to the combined uncertainty was $< 1\%$

Table S5: Nitrate in SPIN-1 (mg/g) by ion chromatography: measurement (standard uncertainty) at NRC

Analysis	SA Cond[a]	SA UV[b]	ExtCal Cond[c]	ExtCal UV[d]
1	21.98 (0.33)	23.58 (0.42)	22.39 (0.23)	23.06 (0.22)
2	21.70 (0.55)	23.12 (0.20)	22.50 (0.15)	22.85 (0.21)
3	21.72 (0.47)	23.29 (0.29)	22.11 (0.24)	22.58 (0.28)
4	22.17 (0.37)	23.88 (0.32)	22.60 (0.14)	22.81 (0.23)
5	21.82 (0.57)	23.41 (0.26)	22.85 (0.44)	23.15 (0.21)
Mean	**21.88 (0.21)**	**23.46 (0.29)**	**22.49 (0.27)**	**22.89 (0.22)**

[a] Standard addition with conductivity detection
[b] Standard addition with UV detection at 210 nm
[c] External calibration with conductivity detection
[d] External calibration with UV detection at 210 nm

S4 Laboratory intercomparison: methods

S4.1 Ion chromatography at NRC lab

A Thermo Scientific ion chromatography system was used for the measurements. A single pump ICS-5000$^+$ SP-5 was interfaced with a AS-AP autosampler (p/n 074925). Detection was performed by suppressed conductivity and by UV. A Thermo Scientific conductivity detector (p/n 061830) was used on-line with a Dionex self-regenerating suppressor (AERS 500, 4 mm, p/n 082540) and UV detection was performed with a Dionex ICS-5 series Variable Wavelength Detector (VWD). Ion separation was achieved with the Dionex IonPack AS23 column (4×250 mm) equipped with the Dionex AG23 guard column (4×50 mm). A 25 µL sample volume was injected and elution was performed in 25 min in isocratic mode (4.5 mM Na_2CO_3, 0.8 mM $NaHCO_3$) at 1.0 mL/min flow rate and 30 °C. A 25 mA current was applied to the suppressor and conductivity detection was attained at 35 °C (20 Hz sample collection rate). The UV chromatogram was collected at 210 nm. For sample preparation 0.2 g of SPIN-1 was extracted in 20 mL of water at 70 °C for 15 min. Sample were then centrifuged (10 min 5000 rpm) and the supernatant was syringe-filtered at 0.45 µm (Phenomenex, nylon membrane 25 mm, p/n AF0-1107-12) and at 0.22 µm (Fisherbrand, PVDF 13 mm, p/n 09-720-3). The filtered solution was then diluted 1:50 in water and analyzed by ion chromatography. Quantitation was obtained by standard addition and external calibration (Table S5).

S4.2 Ion chromatography and ID GC–MS at CNR-ICCOM lab

At the CNR-ICCOM lab, two methods were used for the analysis of SPIN-1, one based on isotope dilution GC–MS[1] and one on ion-chromatography with both conductivity and UV-vis detection. Isotopically enriched nitrate, $K^{15}NO_3$ ($x(^{15}N) = 0.99$ mol/mol) was purchased

Table S6: Nitrate in SPIN-1 (mg/g): measurement (standard uncertainty) at CNR-ICCOM

Analysis	ID GC–MS[a]	IC ExtCal Cond[b]	IC ExtCal UV[c]
1	22.48 (0.04)	22.79 (0.02)	22.83 (0.01)
2	22.50 (0.02)	22.76 (0.08)	22.98 (0.11)
3	22.45 (0.01)	22.75 (0.11)	22.90 (0.02)
4	22.43 (0.03)	23.02 (0.01)	23.01 (0.02)
5	22.48 (0.01)	22.96 (0.10)	22.86 (0.01)
Mean	**22.47 (0.03)**	**22.85 (0.12)**	**22.92 (0.08)**

[a] Primary ID GC–MS (Calibration plot)
[b] External calibration with conductivity detection
[c] External calibration with UV detection

from Cambridge Isotope Laboratories, whereas primary standard solution of nitrate of natural isotopic composition was obtained from Fluka (1001 ± 2 µg/mL NO_3^- in water, analytical standard). Triethyloxonium tetrafluoroborate ($w(Et_3OBF_4) \geq 0.97$ g/g) and sulfamic acid ($w(NH_2SO_3H) \geq 0.99$ g/g) from Sigma-Aldrich were used. Preparation/dilution of samples and solutions was performed gravimetrically using ultrapure water (Elga PureLab; 18.2 MΩ cm at 25 °C).

For the GC–MS sample preparation, five independent SPIN-1 subsamples were prepared as following: 0.2 g of SPIN-1 were spiked with $^{15}NO_3^-$ internal standard and suspended in deionized water (final volume 20 mL). The solution was maintained for 15 min in a water bath at 70 °C, cooled to room temperature and centrifuged (5 min at 5000 rpm). 2 mL of supernatant was derivatized with Et_3OBF_4 as reported previously[1]. A six levels isotope dilution calibration plot was prepared and used for quantitation after fitting with the Padé [1/1] approximant[2] (Table S6).

For the ion chromatography experiments, five independent aliquots were prepared as following: 0.2 g of sample was suspended in deionized water (final volume 20 mL). The solution was maintained for 15 min in a water bath at 70 °C, cooled to room temperature and centrifuged (5 min at 5000 rpm). The supernatant was diluted and injected in the ion chromatography system. Detection was achieved with both conductivity and UV and nitrate quantitation was obtained with a four point external calibration plot (Table S6).

S4.3 Ion chromatography at CNR-ISPA lab

An ion exchange chromatography (Dionex DX120, Dionex Corporation, Sunnyvale, CA, USA) with a conductivity detector was performed as reported by D'Imperio *et al.*[3] For the determination of nitrate contents, five independent SPIN-1 samples of 0.308 g were treated with fresh solution of 3.5 mM Na_2CO_3 and 1 mM $NaHCO_3$ for 30 min in an orbital shaker (50

Table S7: Nitrate in SPIN-1 (mg/g) by ion chromatography: measurement (standard uncertainty) at CNR-ISPA

Analysis	ExtCal Cond[a]
1	26.84 (0.46)
2	27.23 (0.27)
3	26.28 (0.07)
4	25.38 (0.26)
5	25.27 (0.34)
Mean	**26.20 (0.87)**

[a] External calibration with conductivity detection

rpm, at room temperature). After extraction, the samples were centrifuged (12.000 g at room temperature) and filtered by using 0.45 µm (RC) followed by Dionex OnGuard IIP (ThermoScientific) in order to remove organic compounds (phenolic fraction of humic acids, tannic acids, lignins, anthocyanins, and azodyes) from the sample matrix. The resulting solutions were analyzed by ion chromatography on an IonPac AG14 precolumn with an IonPac AS14 separation column (Dionex Corporation). Samples of 25 µL were injected on column by means of a loop valve. For each extraction three independent analyses were performed. The elution was performed using 3.5 mM Na_2CO_3 and 1 mM $NaHCO_3$, the flow rate was 1 mL min^{-1} at 35 °C. Detection was obtained by suppressed conductivity (Dionex AERS 500 4 mm RFIC, 50 mA). The samples were quantified by external calibration against the multi element IC Standard sol. IC-MAN-18 (6E), Lot No: 25.5952804 (Table S7).

S4.4 Ion chromatography at IZSPB lab

At the IZSPB lab, two ion chromatography methods were implemented. The first was a conventional ion chromatography method accredited by ACCREDIA:[4,5] nitrate certified anion standard solutions for ion chromatography (1000 mg L^{-1}) was supplied by Sigma-Aldrich. A 0.5 g portion of the SPIN-1 sample was mixed with 100 mL of ultrapure water and then placed at 70 °C for 5 min. After cooling, the mixture was centrifuged for 5 min at 1500 x g at room temperature. The supernatant was filtered through filter Whatman No. 40, and then about 2 mL was filtered through Anotop 10 LC, 0.2 µm, 10 mm filters prior to chromatographic analysis. The chromatographic determinations were performed on a Dionex DX500 system (Thermo Fisher Scientific, Waltham, USA). A Dionex anion self-regenerating suppressor (ASRS II, 4 mm) was used for the electrochemical suppression at an operative current of 50 mA. Ion separation was obtained on an IonPac AS9-HC column (250 mm × 4 mm i.d., particle size: 13 µm) connected to a precolumn AG9-HC (Dionex Corporation, Sunnyvale, CA). Elution was performed in isocratic mode at a flow rate of 1.0 mL min^{-1}. The

Table S8: Nitrate in SPIN-1 (mg/g) by ion chromatography: measurement (standard uncertainty) at IZSPB

Analysis	ExtCal Cond[a]	Cap IC ExtCal Cond[b]
1	24.3 (2.4)	24.40 (0.63)
2	24.4 (2.4)	24.60 (0.64)
3	24.3 (2.4)	24.10 (0.63)
4	24.7 (2.4)	24.80 (0.64)
5	23.7 (2.3)	25.20 (0.66)
Mean	**24.3 (2.4)**	**24.62 (0.64)**

[a] External calibration with conductivity detection
[b] Capillary ion chromatography external calibration with conductivity detection

mobile phase consisted of 9 mM sodium carbonate. The results have been corrected for the mean recovery of the method (101%). The method uncertainty is 9.7% (Table S8).

The second method was based on capillary chromatography (accreditation in progress by ACCREDIA). The same reference materials and extraction procedure described above were used. The chromatographic determinations were performed on a Dionex ICS-4000 capillary HPIC system (Thermo Fisher Scientific, Waltham, USA). An ACES 300 anion capillary electrolytic suppressor cartridge, set at 10 mA was used. Ion separation was accomplished by using a Dionex IonPac AS11-HC capillary column (250 mm $\times$ 0.4 mm i.d.; Particle size: 9 µm), combined with an elution gradient of KOH from 2 mM to 30 mM (total run time, 38 min). The results have been corrected for the mean recovery of the method (99%). The method uncertainty is 2.6% (Table S8).

Table S9: Inter laboratory study on the SPIN-1. For each method, the SPIN-1 was analyzed 5 times

Lab[a]	Method[b]	Calibration[c]	Primary standard	Nitrate (mg/g)[d]
NRC-1	IC-Cond	5 pt StAdd	NIST SRM 3185	21.88 (0.21)
NRC-2	IC-UV	5 pt StAdd	NIST SRM 3185	23.46 (0.29)
NRC-3	IC-Cond	5 pt ExtCal	NIST SRM 3185	22.49 (0.27)
NRC-4	IC-UV	5 pt ExtCal	NIST SRM 3185	22.89 (0.22)
CNR-ICCOM-1	ID GC–MS	6 pt IDMS Cal	Sigma-Aldrich74246	22.47 (0.03)
CNR-ICCOM-2	IC-Cond	4 pt ExtCal	Sigma-Aldrich74246	22.85 (0.12)
CNR-ICCOM-3	IC-UV	4 pt ExtCal	Sigma-Aldrich74246	22.92 (0.08)
CNR-ISPA	IC-Cond		AccuStandard IC-MAN-18	26.20 (0.87)
IZSPB-1	IC-Cond		Sigma-Aldrich74246	24.3 (2.4)
IZSPB-2	Cap IC-Cond		Sigma-Aldrich74246	24.62 (0.64)

[a] Legend in *Materials and Method*
[b] IC-Cond: ion chromatography with suppressed conductivity detection; IC-UV: ion chromatography with UV detection; ID GC–MS: primary isotope dilution method; Cap IC-Cond: capillary ion chromatography with suppressed conductivity detection.
[c] *n* pt StAdd: *n*-points standard addition; *n* pt ExtCal: *n*-points external calibration; 6 pt IDMS Cal: isotope dilution calibration plot with 6 points[2].
[d] Standard uncertainty is reported in parenthesis.

S4.5 Inter laboratory study: summary of the results

For the calculation of the consensus value of data in Tables S5-S6-S7-S8, the Linear Pool model was chosen[6] and the result was generated with the NIST consensus builder. A value of 23.4 mg/g NO_3^- ($u = 1.5$ mg/g, $u_R = 6.3\%$, $k = 1$) was obtained. A summary of all data is presented in Table S9.

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