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

Enea Pagliano · Juris Meija · Beatrice Campanella · Massimo Onor · Marco Iammarino · Teresa D'Amore · Giovanna Berardi · Massimiliano D'Imperio · Angelo Parente · Ovidiu Mihai · Zoltán Mester

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Abstract A high-precision exact-matching quadruple isotope dilution method (ID^4MS) was employed for the quantitation of nitrate in an air-dried spinach powder Certified Reference Material (CRM). The analyte was extracted in hot water following addition of $^{15}NO_3^-$ internal standard. The blend was then treated with sulfamic acid to remove nitrite and with triethyloxonium tetrafluoroborate to promote aqueous conversion of nitrate into volatile $EtONO_2$. The derivative was analyzed by headspace GC–MS with 3 min elution time. The method performance was validated with a series of tests which demonstrated adequate selectivity and ruggedness. This method supported the development of novel SPIN-1 CRM giving a modest contribution to its uncertainty ($u_{\mathrm{char}} = 0.85\%$). With respect to previous attempts, the SPIN-1 was proven stable, homogeneous ($u_{\mathrm{hom}} = 0.44\%$) and suitable for spinach monitoring under EU regulations. On dried basis, the nitrate content of SPIN-1 was found to be 22.53 ± 0.43 mg/g ($U_{\mathrm{c}} = 1.9\%$, $k = 2$). The material was also used in an inter laboratory study were four laboratories employed a total of ten measurement methods.

Keywords Certified Reference Material · dietary nitrate · leafy vegetable · isotope dilution

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1 Introduction

In the past decades, the health effects of dietary nitrate have been widely discussed in the literature [1, 2]. Despite NO_3^- being relatively inert in its native form, in the human body it can be converted into biologically active molecules. For example, nitrate has been associated with the *in vivo* generation of nitric oxide with benefits to the cardiovascular function [3, 4], blood pressure reduction [5], and possible applications in sport medicine [6]. These positive aspects are counterbalanced by the disposition of dietary nitrate to generate carcinogenic *N*-nitrosamine [1, 7–9] and, to date, the safety and nutritional properties of nitrate are still a matter of ongoing debate. Following the precautionary principle, in 2011 the EU regulated nitrate content of spinach, lettuce, and rucola [10]. These leafy vegetables may contain over 2 g/kg NO_3^- and their consumption can rapidly exceed the Acceptable Daily Intake of nitrate (3.7 mg/kg BW/day NO_3^-, estimated by the Joint FAO/WHO Expert Committee on Food Additives). The amount of residual nitrate in spinach, lettuce, and rucola depends on agricultural practices and its monitoring is required to demonstrate regulatory compliance [11].

Nitrate is present in leafy vegetables at significant levels and it can be detected by standard analytical methods [12]. The extraction of nitrate is normally performed by hot water [13] and detection can be carried out by spectrophotometry [14–16] and electroanalytical methods [17, 18], directly or with the aid of liquid chromatography (LC) [19–23] and electrophoresis [24, 25]. Photometric methods relies on the reduction of nitrate into nitrite. As an example, the ISO 6635:1984 method performs such reduction on a metallic cadmium column and nitrite is detected at 538 nm after reaction with sulfanilamide and *N*-(1-naphthyl) ethylenediamine (Griess chemistry). This approach, however, is not always comparable with LC, and bias up to 60% have been reported [13]. The Achilles' heel of nitrite-based photometric methods is the potential incomplete conversion of NO_3^- to NO_2^- which can be matrix dependent and needs careful control. In recent years, LC has gained popularity for nitrate testing in food matrices, but LC-procedures provide a low sample throughput and they are typically interfaced with non-specific detectors (conductivity or UV) which may lead to chromatographic peaks which are difficult to integrate when complex matrices are analyzed. Selectivity issues are more pronounced for direct electroanalytical detection.

The availability of reference procedures and Certified Reference Materials (CRMs) for dietary nitrate could play an important role for the harmonization of analytical data generated by food testing laboratories. Re-

cently, we developed a primary species-specific isotope dilution mass spectrometry (IDMS) approach for the determination of nitrate in vegetables [26]. The method entails extraction of NO_3^- in hot water, addition of $^{15}NO_3^-$ internal standard, and aqueous conversion of nitrate to volatile $EtONO_2$ by triethyloxonium tetrafluoroborate. The $EtONO_2$ derivative can be sampled from the headspace and analyzed by gas chromatography mass spectrometry (GC–MS) with 3 min of elution time. This procedure is simple, offers high selectivity, and the IDMS calibration ensures high-precision results.

In this study, the IDMS method was selected as a reference technique for characterization of SPIN-1 air-dried spinach powder Certified Reference Material (CRM) [27]. The SPIN-1 preparation is descried along with the validation figures of the primary IDMS method used for value assignment. The nitrate content of the SPIN-1 was proven homogeneous, stable under storage/transport conditions, and suitable for quality control and method development for spinach testing under EU regulations. Results from an inter-laboratory study are also discussed and compared to the SPIN-1 certified property value.

2 Materials and Methods

2.1 Reagents and primary standards

The NIST SRM 3185 (Lot No. 050517) and the Sigma-Aldrich 74246 (Lot No. BCBT3311) were used as primary nitrate standard solutions. The enriched $^{15}NO_3^-$ internal standard was supplied from Cambridge Isotope Laboratories ($K^{15}NO_3$, 99% ^{15}N). Triethyloxonium tetrafluoroborate (Et_3OBF_4, 97%+), sulfamic acid (H_3NSO_3, 97%+), and acetonitrile (MeCN) were from Sigma-Aldrich. Ultrapure water was generated in-house with a Thermo Scientific GenPure UV xCAD plus system (18.2 MΩ cm at 25 °C). A solution of Et_3OBF_4 in MeCN was prepared by adding 5 mL of pre-cooled MeCN (–20 °C) to 5 g of Et_3OBF_4. When stored at –20 °C the solution is stable for several months, but it decomposes at room temperature within few hours. The leftovers should be hydrolyzed before disposal ($Et_3OBF_4 + H_2O \rightarrow Et_2O + H_3O^+ + BF_4^-$). The Et_3OBF_4 is a non volatile salt and a strong alkylating agent that should be used in a fumehood with personal protective equipment.

2.2 Preparation of the SPIN-1 CRM

A 25 kg of food grade air-dried spinach powder was sourced commercially (country of origin: China). The material was transfered in a plastic drum with inner mixing blades and further homogenized for 24 hours on a rotary drum mixer. The material was bottled in 30 mL amber glass bottles (14 g of material per unit), sealed in trilaminate bags, and stored in a freezer at –20 °C.

2.3 Sample preparation and IDMS method

The primary IDMS method used to study the SPIN-1 is discussed in a previous publication [26]. The procedure was adopted for this certification and further validated. A 0.2 g aliquot of spinach powder was transfered in a glass vial with 20 mL of water, shaken, and incubated in a hot block for 15 min at 70 °C. Then, 1 mL of cold digest was transfered in a 15 mL Falcon tube with 0.2 mL of internal standard (1.11 mg/g $^{15}NO_3^-$ in water). The mass of sample (and of internal standard) was recorded. The blend was vortex-mixed and centrifuged (10 min at 5000 rpm). The supernatant (1 mL) was transfered into a 10 mL headspace vial with 1 mL of aqueous 0.1% sulfamic acid. The blend was then treated with 50 µL Et_3OBF_4/MeCN solution (paragraph 2.1) to convert nitrate into $EtONO_2$ which was detected by headspace GC–MS (Figure 1). For the SPIN-1 certification, two instruments were employed: an Agilent 7000 TripleQuad GC–MS with a CTC PAL3 autosampler was used for EI SIM and EI MRM acquisitions whereas a Hewlett-Packard 5973 GC–MS with CTC CombiPAL autosampler was used for PCI SIM and NCI SIM (settings are reported in ESM, paragraph S1).

The quadruple isotope dilution measurement model was used for quantitation [28]:

$$
\begin{aligned}
w_{\mathrm{A}} &= -\frac{z_2 \cdot z_3 \cdot r_4 + z_1 \cdot z_3 \cdot r_5 + z_1 \cdot z_2 \cdot r_6}{z_1 \cdot r_4 + z_2 \cdot r_5 + z_3 \cdot r_6} \cdot \frac{m_{\mathrm{B}}}{m_{\mathrm{A}}} \\
z_i &= w_{\mathrm{A^*\text{-}}i} \cdot \frac{m_{\mathrm{A^*\text{-}}i}}{m_{\mathrm{B\text{-}}i}} \text{ where } i = 1,\ 2,\ 3 \\
r_4 &= (r_{\mathrm{AB}} - r_{\mathrm{A^*B\text{-}1}}) \cdot (r_{\mathrm{A^*B\text{-}2}} - r_{\mathrm{A^*B\text{-}3}}) \\
r_5 &= (r_{\mathrm{AB}} - r_{\mathrm{A^*B\text{-}2}}) \cdot (r_{\mathrm{A^*B\text{-}3}} - r_{\mathrm{A^*B\text{-}1}}) \\
r_6 &= (r_{\mathrm{AB}} - r_{\mathrm{A^*B\text{-}3}}) \cdot (r_{\mathrm{A^*B\text{-}1}} - r_{\mathrm{A^*B\text{-}2}})
\end{aligned}
\tag{1}
$$

The mass fraction of the analyte in the sample (w_{A}) is formally related to the primary standards used for calibration ($w_{\mathrm{A^*\text{-}}i}$, i = 1–3). The calibration blends (A*B-i) were prepared by mixing a mass of primary

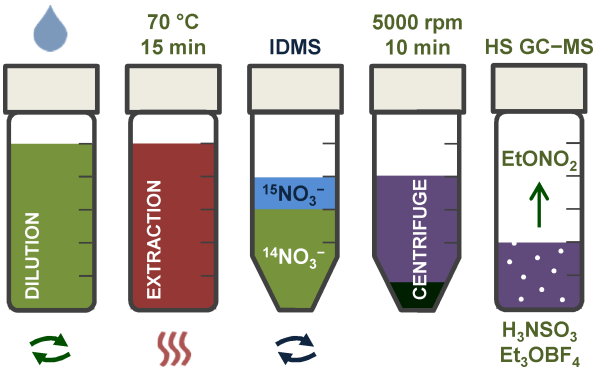

Fig. 1: Outline of the sample preparation used for the GC–MS analysis of SPIN-1 CRM. 0.2 g of sample was diluted in 20 mL of water and extracted at 70 °C for 15 min. The ^{15}N-nitrate internal standard was added and after equilibration the nitrate was converted to $EtONO_2$ and analyzed by headspace GC–MS.

standard ($m_{A*\text{-}i}$) with a mass of $^{15}NO_3^-$ internal standard ($m_{B\text{-}i}$). Similarly, the sample blend was prepared by mixing a mass of sample (m_A) with internal standard (m_B). A Mettler Toledo XPE205 balance with antistatic kit was used for all preparation/dilution. In addition to gravimetric data, Eq. 1 requires the measured isotope ratios (r) of the calibration blends ($r_{A*B\text{-}i}$, n = 1–3) and of the sample blend (r_{AB}). In the case of $EtONO_2$, the r could be measured in five independent modes. In EI SIM we monitored ion ratios m/z 46:47 ($^{14}NO_2^+/^{15}NO_2^+$) and 76:77 ($CH_2O^{14}NO_2^+/CH_2O^{15}NO_2^+$). In EI MRM the m/z transitions 76→46 and 77→47 were acquired and used for the calculation of r. In PCI and NCI SIM we monitored ion ratio m/z 92:93 ($EtO^{14}NO_2H^+/EtO^{15}NO_2H^+$) and ion ratio 46:47 ($^{14}NO_2^-/^{15}NO_2^-$), respectively. In each case, the numerical value of r was obtained by dividing the chromatographic peak area extracted at the most abundant ion in the sample (*e.g.* m/z 46 in NCI SIM) over the one most abundant ion in the internal standard (*e.g.* m/z 47 in NCI SIM). For best results, Eq. 1 was applied on the exact-matching design [28], where the sample blend was prepared to yield $r_{AB} \approx 1$, one of the calibration blends was matching this ratio ($r_{A*B\text{-}2} \approx 1$), and the other two were bracketing it ($r_{A*B\text{-}1} \approx 0.5$ and $r_{A*B\text{-}3} \approx 2$).

2.4 Uncertainty calculation

Uncertainty of the mass fraction of nitrate in SPIN-1, $u(w_A)$, was evaluated by classical uncertainty propagation (Gauss method) as outlined in the JCGM 100:2008 guidelines. The 15 variables of Eq. 1 ($w_A = f(x_i)$) were

treated as uncorrelated and the combined standard uncertainty was calculated using the following equation:

$$u(w_{\mathrm{A}}) = \sqrt{\sum_{i=1}^{15} \left(\frac{\partial f(x_i)}{\partial x_i} \right)^2 \cdot u^2(x_i)} \tag{2}$$

The measurement model relies on measured masses of aqueous solutions (m), isotope ratios (r), and on the mass fraction of nitrate in the primary standards ($w_{\mathrm{A}*}$). Based on repeated observations, the uncertainty on weighing of aqueous solutions was estimated to be 0.0001 g and all readings were corrected for buoyancy. Furthermore, the mass difference observed on the extraction vial after the thermal treatment (Fig. 1) was added to the uncertainty of the mass of the solution. Since the vials were not perfectly air tight, such minor differences were due to the evaporation occurring at 70 °C. For the estimation of the uncertainty on r, each sample/standard blend was analyzed three times and the standard deviation was used as an estimate of $u(r)$. The uncertainty of the mass fraction of the primary standard was provided by the vendor (NIST and Sigma-Aldrich). An example of calculation with Eq. 1 along with the uncertainty propagation is given in the ESM.

2.5 Homogeneity evaluation

To account for small variations during sample preparation, the observed isotope ratios (r_{AB}) were normalized by the gravimetric data:

$$S_{\mathrm{AB}} = r_{\mathrm{AB}} \cdot \frac{m_{\mathrm{B}}}{m_{\mathrm{A}}} \tag{3}$$

For homogeneity assessment and for some validation experiments, S_{AB} was used to estimate repeatability standard deviations. Hence, the analytical response was compared directly in a single run experiment without the need for calibration (Eq. 3). The blends of sample (m_{A}) and internal standard (m_{B}) were prepared gravimetrically and measured by GC–MS (Fig. 1). The S_{AB} value was used to directly compare samples analyzed in a single run. This normalization is strictly correct only with no overlap between the isotopes of sample and internal standard. However, when this overlap is modest and with small difference in the gravimetric composition of the blends, this simplification is acceptable. For the SPIN-1 homogeneity study, the added uncertainty due to simplifications introduced to Eq. 3 was only 0.03%.

2.6 The interlaboratory study

Besides the analyses performed at the National Research Council Canada (NRC) for the certification of nitrate in SPIN-1, the material was also tested in an inter laboratory study which involved NRC, two laboratories at the Consiglio Nazionale delle Ricerche (Istituto di Chimica dei Composti Organometallici, CNR-ICCOM; Istituto di Scienze delle Produzioni Alimentari, CNR-ISPA), and the Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata (IZSPB). The experimental details about the methods employed in the study are presented in the ESM.

3 Results and Discussion

3.1 Validation of the IDMS method

Validation experiments including spike recovery tests, comparison with ion chromatography, and internal standard equilibration were discussed previously [26] along with the performance characteristic of the IDMS method. In this study, the method was further validated with a series of robustness tests which demonstrated the fitness of the procedure for the certification of SPIN-1. The novel evidences demonstrated quantitative extraction of nitrate from the SPIN-1 matrix in hot water and confirmed the high-selectivity of the detection system.

3.1.1 Aqueous extraction of nitrate from SPIN-1

Aqueous extraction at 70 °C for 15 min allowed quantitative recovery of nitrate from the spinach powder matrix (Fig. 1) [26]. The validity of this extraction was proven by robustness experiments. Initially, we investigated the effect of the sample-to-water ratio. Five 0.2 g SPIN-1 aliquots were extracted with 5-10-20-30-40 mL of water at 70 °C for 15 min. No difference in the response was observed demonstrating that variations in the extraction volume had no impact on the results (ESM, Fig. S1).

Most of the published methods for nitrate in vegetables require aqueous analyte extraction, whereas other methods (ISO 6635:1984), employ an alkaline aqueous solution of disodium tetraborate decahydrate (borax) instead. The effect of pH for nitrate extraction was investigated. The SPIN-1 was extracted multiple times in water, 2.5 g/L borax, and 4 g/L NaOH at 20 °C, 70 °C, and 100 °C for 30 min. As reported in Fig. 2a, the extraction efficiency of all tested media was indistinguishable in all conditions. A minor variation of the

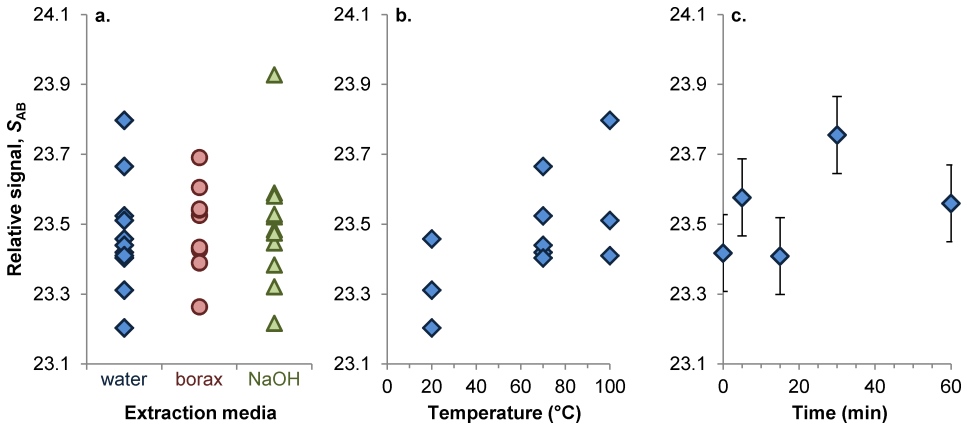

Fig. 2: Robustness test on the nitrate extraction. a. Effect of the extraction media: 0.2 g of SPIN-1 was extracted with 20 mL of water (blue diamonds), 2.5 g/L borax (red circles), and 4 g/L NaOH (green triangles) at various temperature (20-70-100 °C). b. Effect of extraction temperature: 0.2 g of SPIN-1 was extracted with 20 mL of water for 30 min at 20-70-100 °C. c. Effect of extraction time: 0.2 g SPIN-1 were extracted in 20 mL of water for 0-5-15-30-60 min.

extraction efficiency was observed by increasing the temperature from 20 to 70 °C (Fig. 2b) and no effect was detected by increasing the extraction time from 0 to 60 min (Fig. 2c). Notably, during hot block extraction, minor water losses due to evaporation were observed at 100 °C. For the SPIN-1 certification, the mass of the solution was measured before and after extraction (70 °C for 15 min) and the difference was incorporated into the combined uncertainty.

Finally, we performed sequential extractions of the 0.2 g aliquot of SPIN-1. After the first extraction, the SPIN-1 solution was centrifuged, 1 mL of supernatant was collected for analysis and the remaining was paper filtered on Buckner funnel. The residue was extracted two more times in water (70 °C, 15 min) and the three extracts were analyzed by GC–MS. The absolute nitrate signal (EI SIM at m/z 46) from the second extraction was only 1.5% relative to the first one, and no nitrate was detected in the third extract.

These tests demonstrate that major variations of the extraction conditions, including chemistry of the media, temperature, time, and sample-to-solvent ratio, have no effects on the recovery of nitrate from SPIN-1 sample. These results confirm that complete extraction can be obtained in hot water as described by others [13].

Farrington *et al.* [13] reported that cold water extraction from spinach may give inconsistent results. Notably, near complete extraction of nitrate from SPIN-1 could be obtained in cold water (Fig. 2b). This suggests that nitrate in the dried powder matrix may be more available toward extraction than in the fresh matrix.

154 *3.1.2 Natural isotopic composition of nitrate in SPIN-1 and primary standards*

155 In isotope dilution equations, such as the Eq. 1, identical isotopic composition of sample (r_A) and primary
156 standard (r_{A*}) is assumed [28, 29]. When performing metrological work, this simplification should be verified.
157 As reported in Tables S1-S2 (ESM), the isotope ratios of all natural materials (r_A and r_{A*}) were measured
158 five times in all detection modes. In our case, the mismatching between (r_A) and (r_{A*}) translated in a bias on
159 the measurement result <0.3%. Such an effect was included in the evaluation of the SPIN-1 characterization
160 uncertainty (u_{char}).

161 *3.1.3 MS detection, selectivity, and procedural blanks*

162 The sample preparation was based on the aqueous conversion of NO_3^- into volatile $EtONO_2$ which was sampled
163 from headspace and analyzed by GC–MS. The detection of $EtONO_2$ was obtained in EI, PCI, and NCI
164 mode and for each sample five independent isotope ratios r_{AB} were obtained (paragraph 2.3). The $EtONO_2$
165 derivative eluted within 3 min of isotherm at 30 °C and the chromatograms collected on the SPIN-1 sample
166 are reported in Fig. 3. As expected, the procedural blanks did not have any impact on this determination and
167 the chromatography was very clean in all cases. Only the signal at m/z 93 in PCI SIM was affected by a high
168 baseline. For each sample, the quadruple isotope dilution equation (Eq. 1) was applied using all five measured
169 isotope ratios. All results were identical regardless of the acquisition mode (Fig. 4a, and ESM Table S3). This
170 highlights the high-selectivity of the IDMS method whose performance is not affected by large variations in
171 the detection strategy. Such selectivity is largely due to the sample preparation procedure which allowed a first
172 major separation of the analyte from a complex food matrix to headspace. A second separation was obtained
173 by gas chromatography and, at last, a third level of separation was achieved by mass spectrometry detection.

174 3.2 Production and certification of the SPIN-1

175 *3.2.1 Format, stability and homogeneity*

176 In 2004 Castanheira *et al.* [30] described the preparation of an in-house reference material for the monitoring
177 of nitrate in vegetable matrices. The authors prepared wet purées of various vegetables and monitored their
178 short-term stability at various temperatures. When the purées were stored for more than a week at 4 °C or

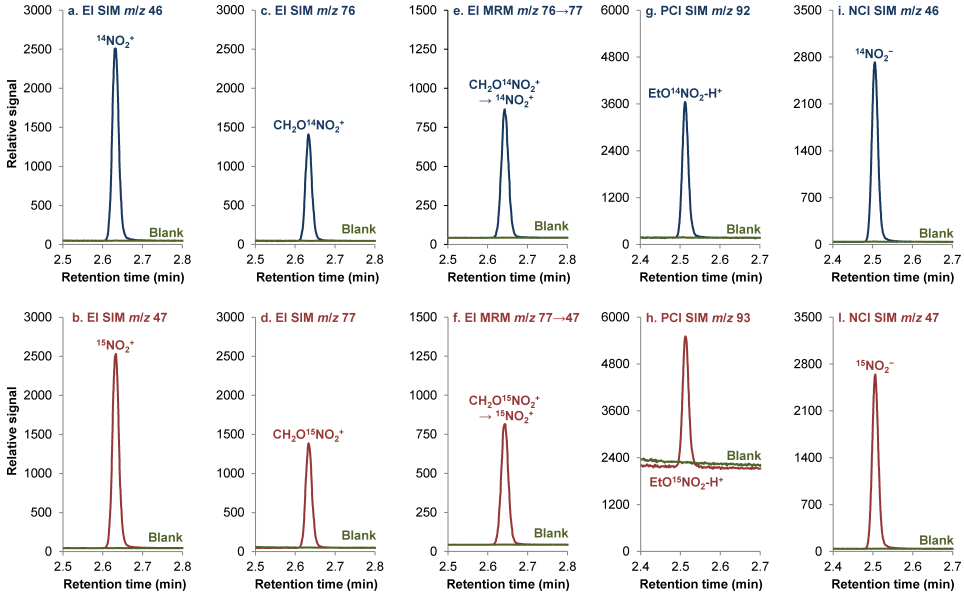

Fig. 3: Gas chromatography of the SPIN-1 mixed with the ^{15}N-nitrate internal standard. The $EtONO_2$ derivative was detected by mass spectrometry in several ionization modes. Top row: signals of the ions containing ^{14}N (most abundant in the SPIN-1 sample). Bottom row: corresponding signals containing ^{15}N (most abundant in the internal standard). In each mode, the isotope ratios were obtained by dividing the peak area of the ^{14}N signal (top row) by the corresponding ^{15}N signal (bottom row). In all cases, analysis of the procedural blank resulted in a clean baseline.

higher, their nitrate content decreased significantly [30]. Notably, the authors found that the spinach purée was stable only when stored at –70 °C [30]. Preparation, storage and transportation of a CRM which requires to be kept at –70 °C involves complex logistic. Hence, we explored an alternative format able to yield more stable CRM. Considering that inorganic nitrate salts are thermally and chemically stable under normal conditions, we ascribed the nitrate instability reported by Castanheira *et al.* [30] to biological activity occurring in the wet samples. For this reason, we studied the short term stability of nitrate in the SPIN-1 air-dried spinach powder (paragraph 2.2) under typical transportation conditions. Six units of SPIN-1 were withdrawn from the storage freezer at –20 °C and analyzed by IDMS for nitrate. Thereafter, set of two bottles were stored at –20 °C, 20 °C, and 40 °C for 20 days before nitrate testing. Even though this stress test used more severe conditions than the one describe by Castanheira *et al.* [30], no nitrate depletion was observed on the dried spinach powder (Fig. 5a). The homogeneity of the SPIN-1 was then evaluated under repeatability conditions. 21 units of SPIN-1 were uniformly sampled across the lot (1434 units total) and analyzed by NCI GC–MS following the preparation in Fig. 1. We assumed negligible within-unit inhomogeneity and we analyzed a single unit 7 times in order to estimate the relative repeatability standard deviation (RSD = 0.32%, $n = 7$). Thereafter, the 21 units were analyzed in a randomized sequence. The relative uncertainty due to between-unit homogeneity was estimated

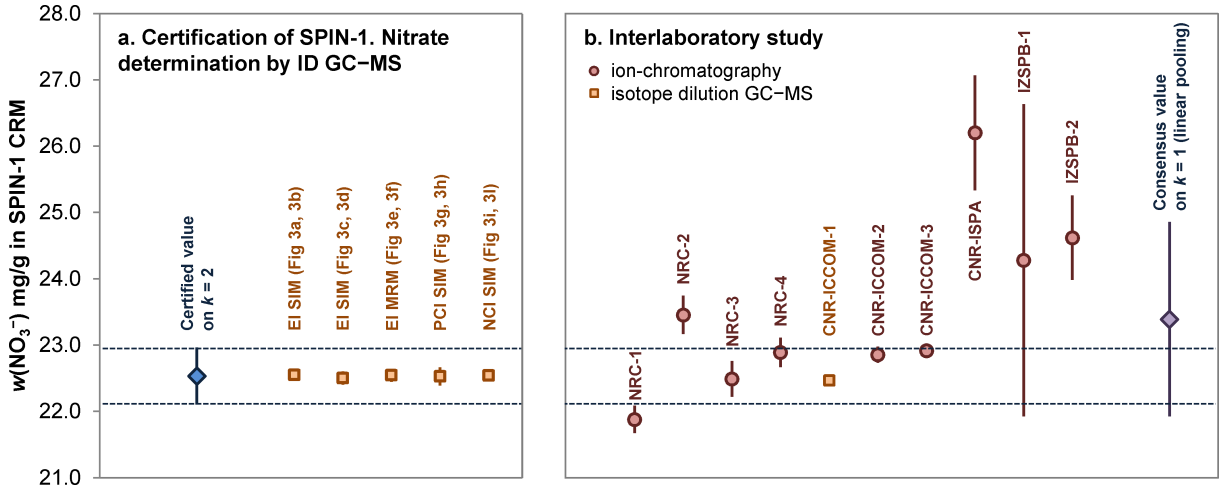

Fig. 4: Comparison of data generated by different methods. a. Certification of nitrate in SPIN-1 CRM by ID GC–MS at NRC; results generated using various acquisition modes (Fig. 3) were identical. b. An inter laboratory study where the SPIN-1 was analyzed by four laboratories with multiple methods (ESM, Table S9).

using the Bayesian ANOVA approach [31] and the conservative upper bound was adopted, $u_{\mathrm{hom}} + 2u(u_{\mathrm{hom}})$, instead of the best estimate, u_{hom}. On 0.2 g sample size we obtained $u_{\mathrm{hom}} + 2u(u_{\mathrm{hom}}) = 0.44\%$ (Fig. 5b). Under recommended storage conditions (–20 °C) the SPIN-1 was also tested for a period of 6 months; no instability in the measured nitrate content of SPIN-1 was observed (Fig 5c).

The residual moisture content in the SPIN-1 was measured by mass loss after drying in a vacuum oven. For this purpose, five sub-samples (0.75 g) from two CRM units were kept in the vacuum oven (20 °C, 25 mm Hg) for 14 days. The average mass difference before and after drying was 1.2%. As expected, this dry material is hygroscopic, showing a significant mass gain (up to 6%) when kept uncapped (Fig. 5d). Hence, sampling of the SPIN-1 was carried out within few seconds and the unit was tightly sealed to protect the bulk material from ambient moisture. Within the conditions employed to generate Fig. 5d, a weighing time of 1 min can result in a mass gain on the sample of 0.1%. For high-precision work, Fig. 5d could be employed to account for the moisture adsorption of SPIN-1.

3.2.2 Value assignment by IDMS

Value assignment for the mass fraction of nitrate in SPIN-1 was performed by relying on the primary species-specific isotope dilution GC–MS method (paragraph 2.3). On the first day of analysis, a single unit of SPIN-1 was analyzed 7 times and 20 additional units were analyzed, each one time. On the second and third day, 15 units were analyzed one time each (paragraph 3.2.1). All selected units were analyzed by GC–MS in all

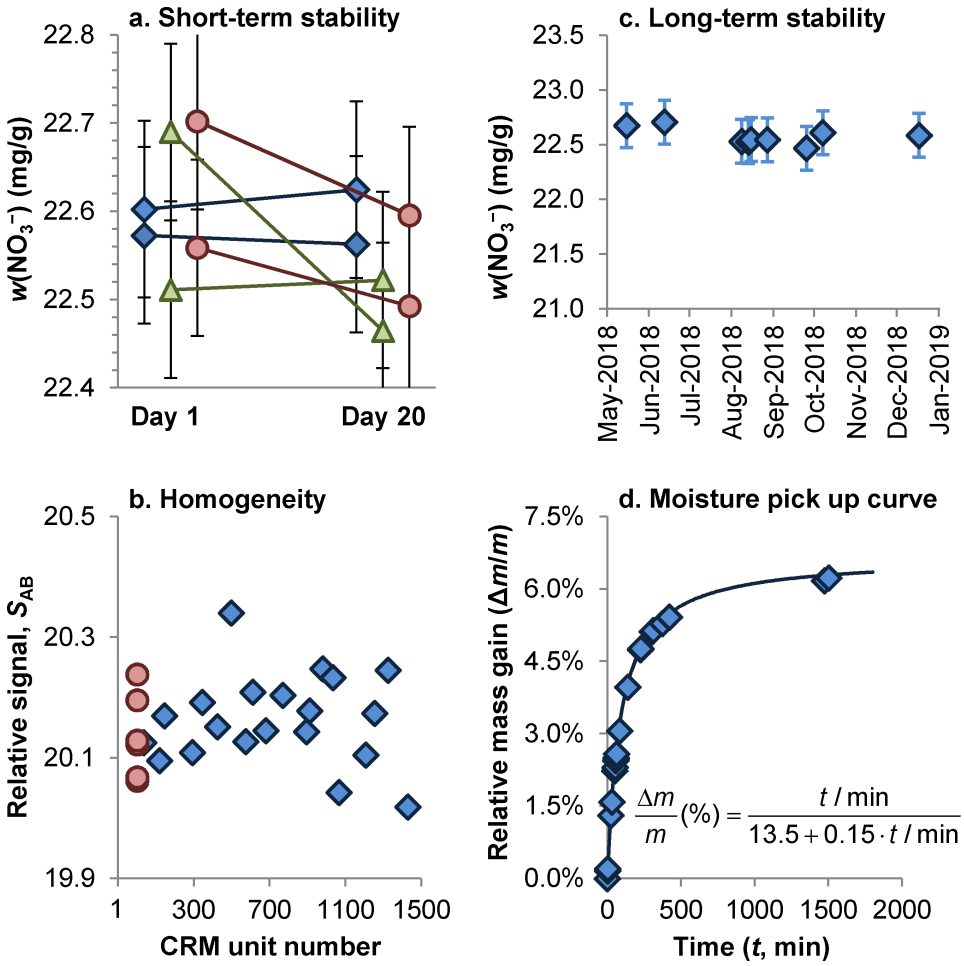

Fig. 5: Properties of the SPIN-1 CRM. a. 20 days short-term stability study under transportation condition: –20 °C (blue diamonds), 20 °C (green triangles), and 40 °C (red circles). b. Homogeneity study under repeatability condition (n = 21, 0.2 g sample size, blue diamonds); the method standard deviation was estimated from repeated measurement of the same unit (n = 7, red circles). c. 6 months long-term stability study under storage condition (–20 °C). d. 0.5 g of SPIN-1 was spread in an aluminum weighing boat and the mass increase due to moisture was monitored *vs* time; the mass measurements were performed at 21 °C in 55–65% relative humidity environment.

ionization modes (Fig. 3) in triplicate and the average isotope ratio and its standard deviation were used for the quantitation (Eq. 1, ESM). Two set of calibration solutions were prepared for the exact-matching quadruple isotope dilution quantitation (Eq. 1, Fig. 6a). The first set of calibrants was prepared by direct dilution of the NIST SRM 3185, while the second set was obtained from the Sigma-Aldrich 74246 nitrate standard whose traceability was established based on SRM 3185. As expected, the results obtained against the two set of standards were identical as their average ratio did not differ significantly from 1 (1.0011 ± 0.0042, $n = 57$). As part of the characterization study, 57 subsamples of SPIN-1 were analyzed and for each of them 10 results were then generated: 5 ionization modes × 2 set of calibrants. The distribution of all 570 quantitative results is shown in Fig. 6 with an average of $w(\mathrm{NO_3^-}) = 22.53$ mg/g and standard deviation of 0.10 mg/g. A full bottom-up

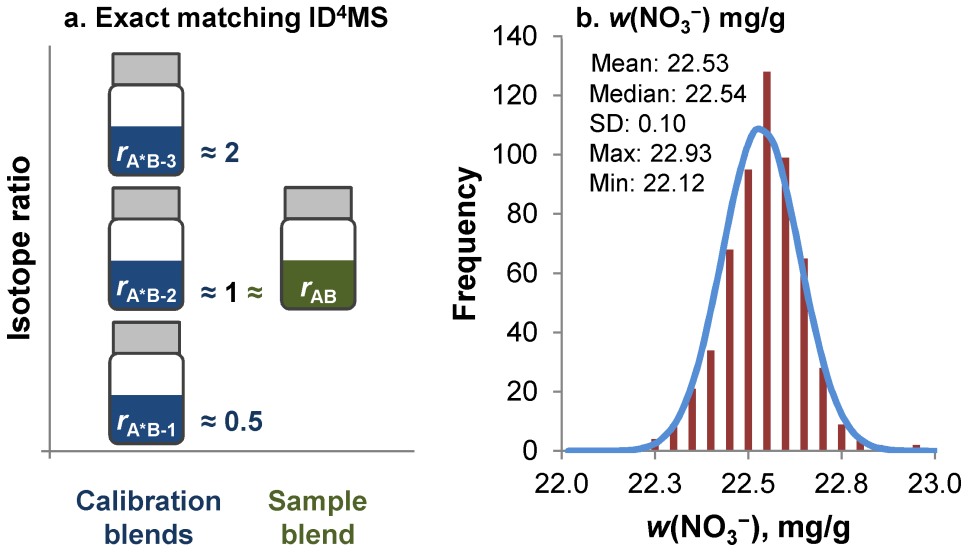

Fig. 6: Characterization of SPIN-1 by IDMS. a. Quadruple isotope dilution calibration: the exact-matching design [28]. b. Distribution of the nitrate mass fraction results obtained for SPIN-1 (n = 570).

error propagation on Eq. 1 was used for the estimation of the uncertainty component due to characterization (u_{char}). For SPIN-1 we found u_{char} = 0.19 mg/g NO_3^- (0.85%). This value included the random effects due to measurement, the uncertainty on the primary standards, and the model limitation discussed in paragraph 3.1.2. Notably, as shown in Table S4 (ESM) the variance components that mostly contributed to u_{char} where the ones associated with the measured isotope ratio of the sample blend $u(r_{\mathrm{AB}})$, the measured isotope ratio of the exact-matching calibrant $u(r_{\mathrm{A*B\text{-}2}})$, and the mass fraction of the primary standard $u(w_{\mathrm{A*}})$. Considering the stability of nitrate in SPIN-1 (paragraph 3.2.1) and the estimated uncertainty on u_{char} and u_{hom}, the proposed certified property value for $w(NO_3^-)$ in SPIN-1 is 22.53 ± 0.43 mg/g (U_{R} = 1.9%, k = 2). The summary of the uncertainty components is reported in Table 1.

Table 1: SPIN-1 CRM: value assignment and uncertainty components

Variable[a]	Value
$w(NO_3^-)$	22.53 mg/g
U_c (k = 2)	0.43 mg/g (1.9%)
u_c	0.95%
u_{hom}	0.44%
u_{char}	0.85%
u_{stab}	0.00%

3.2.3 Use of the SPIN-1 CRM

Although the dry format chosen for the SPIN-1 was essential to obtain stability, one must consider that the typical water content in fresh spinach is ≈90% [32]. When the water is removed from the fresh material, a tenfold preconcentration of analytes and matrix components is observed on the dry powder [33]. This effect should be taken into account when the SPIN-1 is used for quality control. Hence, the amount of SPIN-1 to be extracted/analyzed by a standard procedure should be one-tenth of the mass of fresh spinach leaves required by the method. In this way, the SPIN-1 extract will match a matrix of a fresh spinach sample containing approximately 2200 mg/kg nitrate which is well suited for quality control of methods employed for testing spinach against the Commission Regulation (EU) No 1258/2011. This European law sets limit of 2000 mg/kg nitrate in preserved, deep-frozen or frozen spinach and of 3500 mg/kg in fresh spinach.

As reported in paragraph 3.1.1, the extraction of nitrate from the dry spinach powder might require milder conditions compared to the fresh matrix. This effect should be considered when the SPIN-1 is used for method development. In this case, the extraction procedure should also be optimized on the fresh matrix through robustness experiments, for example, as described in paragraph 3.1.1.

3.3 The SPIN-1 in an inter-laboratory study

The SPIN-1 CRM was also analyzed in an interlaboratory study (paragraph 2.6). Four laboratories each analyzed a single unit of SPIN-1 using multiple methods and the results are reported in Fig. 4 and summarized in ESM (Table S9). For each method, the same unit of SPIN-1 was analyzed 5 times and the raw data are reported in the ESM (paragraph S4, Tables S5 to S8). For the calculation of the consensus value, the Linear Pool model was chosen [34]. The NIST consensus builder (https://consensus.nist.gov/) was used for calculation and resulting consensus was 23.4 mg/g NO_3^- (u = 1.5 mg/g). Despite the consensus value was mostly driven by ion chromatography methods, this value is compatible with the certified value based on isotope dilution 22.53 ± 0.43 mg/g (U_R = 1.9%, k = 2).

4 Conclusion

The potential adverse effects of dietary nitrate on human health have motivated the European legislator to set limits for nitrate content in certain leafy vegetables including spinach. Methodologies mostly used for nitrate monitoring include ion chromatography with suppressed conductivity, photometry based on Griess chemistry, and direct detection with ion-selective electrodes and sensors. Since the data generated by these techniques are not always reliable, the availability of reference methods and Certified Reference Materials (CRMs) may contribute to improve standardization of nitrate measurements in food matrices. In this study, we describe the implementation of a high-precision isotope dilution method for the certification of nitrate in a new air-dried spinach powder CRM (SPIN-1). The validity of the primary method was demonstrated with robustness experiments and its application to the SPIN-1 allowed nitrate certification within 2% expanded uncertainty. Notably, the dried format chosen for the SPIN-1 CRM avoided the biological degradation of nitrate reported for purée-based materials. We believe that this CRM may contribute to the harmonization of analytical data and methods used to demonstrate compliance with the Commission Regulation (EU) 1258/2011.

5 Conflict of Interest

The authors declare no conflict of interest.

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