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Determination of elevated levels of nitrate in vegetable powders by high-precision isotope dilution GC–MS

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Abstract

A high-precision isotope dilution GC–MS method was employed for the determination of nitrate in processed vegetables. The samples were extracted in water, derivatized with triethyloxonium tetrafluoroborate and analyzed by headspace GC–MS (15 samples/hour). The method was applied to estimate the effect of drying on the content of nitrate in vegetables. The absolute amount of nitrate in a spinach sample before and after drying did not change even when the material was baked at 105 °C. Elevated levels of nitrate were found in commercial vegetable powders where the nitrate mass fraction exceeded the percent level: 1.2-2.3% NO_3^- was found in spinach powders, 1.3-1.6% in kale powders, and 1.4% in a beetroot powder. The likely reduction of the antioxidant properties seen in vegetable powders along with their high nitrate content suggest the need to study the risk of endogenous *N*-nitrosation associated with these products.

Keywords: food safety, dietary nitrate, vegetable powders

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1. Introduction

The role of dietary nitrate in human health is the center of a debate which is currently dividing many scientists. To date, there is no consensus whether inorganic nitrate should be considered as an essential nutrient (Weitzberg and Lundberg, 2013) or as a food contaminant with potential adverse effects (Santamaria, 2006), or both depending on concentration. The evidence in favor of a diet rich in nitrate are rooted to the biological role of nitric oxide. The *in vivo* generation of NO is essential for life (Culotta and Koshland Jr., 1992) and its genesis through the nitrate-nitrite-nitric oxide pathway is relevant in physiology and therapeutics (Lundberg et al., 2008). Inorganic nitrate is seen as a reservoir for the biosynthesis of nitric oxide and dietary nitrate is perceived as a means to sustain NO production with benefits to the cardiovascular function (Webb et al., 2008; Hord, 2011; Lidder and Webb, 2013; Bryan, 2018). On the other hand, high consumption of nitrate through diet and drinking water has been associated with a number of long term effects including higher risks to develop certain types of cancer (Kim et al., 2002; DellaValle et al., 2013; Inoue-Choi et al., 2015; Espejo-Herrera et al., 2016; Jones et al., 2016) and thyroid diseases (Ward et al., 2010). Notably, in 2010 the International Agency for Research on Cancer (IARC) classified ingested nitrate as a probable human carcinogen under conditions that result in endogenous nitrosation (IARC, 2010).

The biological effects ascribed to dietary nitrate are derived from the complex chemistry of NO_3^- in the human body which starts in the mouth with its reduction to NO_2^- . Thereafter, most of the concerns are focused around the digestive system where formation of *N*-nitroso compounds may occur as the result of nitrite interaction with suitable precursors

23 (Kobayashi, 2018). When the NO_2^- meets secondary amines in the acid stomach, formation
24 of carcinogenic *N*-nitrosamine may occur:



26 Vermeer et al. (Vermeer et al., 1998) demonstrated that the urinary level of certain ni-
27 trosamines tripled when a control diet low in nitrate was followed by the consumption of
28 nitrate at the Acceptable Daily Intake (ADI) level with a fish meal rich in amines. In an-
29 other *in vivo* experiment, Iijima et al. (Iijima et al., 2002) demonstrated that ingestion of
30 an amount of nitrate equivalent to that found in a salad portion may elevate the concentra-
31 tion of NO above 50 $\mu\text{mol/L}$ at the gastroesophageal junction with risk of mutagenesis at
32 this site.

33 Based on potential adverse effects, an ADI for dietary nitrate was established in 2002 by
34 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at the level of 3.7 mg
35 NO_3^- per kg of body weight, meaning that a 60 kg human should limit exposure to 222 mg
36 nitrate per day (Alexander et al., 2008) in order to avoid appreciable health risks associated
37 with nitrate. Vegetables are the major source of dietary nitrate (Santamaria, 2006). While
38 consumption of vegetables is generally accepted to be beneficial for human health, nitrate
39 content may vary by more than tenfold between different vegetable species (Santamaria,
40 2006; Bahadoran et al., 2016). Hord et al. (Hord et al., 2009) showed that a vegetarian
41 high-nitrate diet may yield up to 1222 mg NO_3^- per day (five times above the ADI for a
42 60 kg human) while a vegetarian low-nitrate diet may yield just 174 mg NO_3^- per day (be-
43 low the ADI for a 60 kg human). Dietary habits, therefore, play a crucial role for nitrate
44 exposure and its effects are strongly dependent upon combination with other foods, gut con-

45 ditions (Kobayashi, 2018), and the presence of nutrients like Fe^{2+} and antioxidants (de La
46 Pomélie et al., 2017). Despite some authors considering the current ADI too conservative
47 with respect to the potential benefits that nitrate may provide to the cardiovascular sys-
48 tem (Hord, 2011; Bryan, 2018), a fair recommendation balancing all *pros* and *cons* is still
49 a matter of debate and future studies are needed (Lidder and Webb, 2013; Weitzberg and
50 Lundberg, 2013).

51 In this regard, one aspect of the story that has seldom been considered is the effect of
52 cooking and food processing on dietary nitrate. Prasad and Chetty (Prasad and Chetty,
53 2008) proved that frying leafy vegetables may elevate their nitrate concentration by up to
54 a factor of three. Notably, a study published in 2002 (Kim et al., 2002) established a sig-
55 nificant increase in incidences of gastric cancer correlated with the intake of cooked spinach
56 which is notorious for its high residual nitrate content (Santamaria, 2006). Food process-
57 ing and cooking may be responsible for the degradation of nutrients retained essential to
58 quench *N*-nitrosation, and may contribute to the concentration of nitrate to levels favorable
59 to speeding up the kinetics of unwanted processes (de La Pomélie et al., 2017).

60 In this study, we applied a high-precision isotope dilution headspace GC-MS method (Cam-
61 panella et al., 2017) to evaluate the effect of drying on the nitrate content of vegetables.
62 Spinach was chosen as model and a gentle freeze-dry technique was compared to a severe
63 static thermal process at 105 °C for 48 hours. In both cases, no nitrate degradation was
64 observed. Consequently, several vegetable powders for domestic use were analyzed and the
65 ones derived from high-nitrate vegetables (kale, spinach, and beetroot powders) had a ni-
66 trate content above the percent level. Considering that some industrial drying processes, like

67 the convective drying at high temperature, may be responsible for degradation of incurred
68 antioxidants (Karam et al., 2016) more research is needed to better understand the potential
69 risks of endogenous *N*-nitrosation related to the domestic use of vegetable powders.

70 **2. Materials and methods**

71 *2.1. Reagents*

72 Primary nitrate standard was sourced from Sigma-Aldrich (P/N 74246, Nitrate Standard
73 *TraceCERT*[®], $1001 \pm 4 \mu\text{g/g NO}_3^-$ in water). Isotopically enriched internal standard
74 K^{15}NO_3 was purchased from Cambridge Isotope Laboratories (P/N NLM-765-PK, 99% of
75 ^{15}N enrichment). Triethyloxonium tetrafluoroborate (P/N 90520), acetonitrile (P/N 01324),
76 and sulfamic acid (P/N 481505) were obtained from Sigma-Aldrich. All preparations used
77 ultrapure water generated by a Thermo Scientific GenPure UV xCAD plus system (18.2 M Ω
78 cm at 25 °C).

79 *2.2. Triethyloxonium tetrafluoroborate solution*

80 Triethyloxonium tetrafluoroborate ($\text{Et}_3\text{O}^+[\text{BF}_4]^-$) is a commercially-available alkylating
81 agent which has been employed for the conversion of several inorganic anions into volatile
82 derivatives for GC-MS analysis (Pagliano et al., 2018). A solution of this non-volatile salt
83 was prepared by dissolving 5 g of reagent in 5 mL of acetonitrile pre-cooled to -20 °C. When
84 stored at -20 °C the triethyloxonium tetrafluoroborate solution was stable for more than a
85 month. All manipulations of the reagent were carried out in a fumehood and leftovers were
86 hydrolyzed before disposal.

87 2.3. Nitrate determination by GC-MS

88 We recently validated a novel high-precision approach for the determination of nitrate in
89 vegetables based on isotope dilution headspace GC-MS (Campanella et al., 2017). The pro-
90 cedure employed in this study was adapted from this method and is illustrated in Fig. 1.
91 Briefly, the vegetable sample (2.0 g aliquot if fresh, 0.2 g if dried) was transferred to a 20 mL
92 glass vial (O.D. 28 mm; height 58 mm) followed by the addition of 20 mL of water, vortex
93 mixing, and incubation at 100 °C for 30 min in a hot block. Hot water extraction is a com-
94 mon practice for nitrate determination in vegetables (Farrington et al., 2006). After cooling
95 to room temperature, a 1.5 mL volume of digest was transferred to a 15 mL Falcon tube and
96 300 µL of aqueous 0.959 mg/g $^{15}\text{NO}_3^-$ internal standard was added (for samples with less
97 than 1.2 mg/g NO_3^- only 100 µL of internal standard was added). The blend was vortexed
98 and centrifuged at 5000 rpm for 10 min (Thermo Scientific, Sorvall Legend X1R). A 1 mL
99 volume of supernatant was transferred to a 4 mL glass vial (O.D. 14 mm; height 46 mm)
100 equipped with open-top cap and PTFE/silicon septum for headspace sampling. After the
101 addition of 1 mL of aqueous 0.1% sulfamic acid, the sample was treated with 50 µL of tri-
102 ethyloxonium tetrafluoroborate solution (paragraph 2.2). Such reagent converted $\text{NO}_3^-(\text{aq})$
103 into volatile EtONO_2 which readily separated from the complex matrix under gaseous form
104 and could be sampled from the headspace. A 100 µL headspace aliquot was manually with-
105 drawn with a gas-tight syringe at room temperature and analyzed by GC-MS. For utmost
106 precision, all preparations were achieved gravimetrically on an analytical balance (Mettler
107 Toledo, MS204S/03) and isotope dilution was employed for quantitation (Pagliano et al.,
108 2015). A calculation example is given in the supporting information.

109 For quality control, each sample was measured in triplicate over three days. For each batch,
110 a calibration curve was acquired and one in-house reference material was analyzed. Quan-
111 titation was obtained using the isotope ratio at m/z 46 over 47. In order to verify the
112 specificity, all results were also qualified against the isotope ratio at m/z 76 over 77.

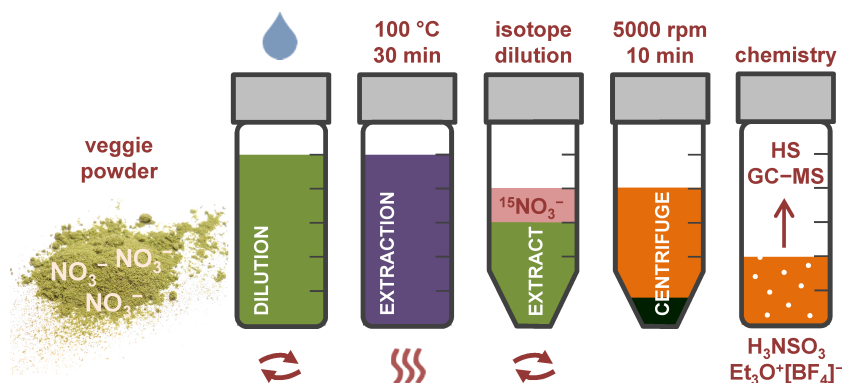


Figure 1: Outline of the sample preparation method for isotope dilution headspace GC-MS determination of nitrate in processed vegetable powders. 0.2 g of sample was diluted with 20 mL of water, vortexed, and digested at 100 °C for 30 min. 1.5 mL digest was mixed with ¹⁵NO₃⁻ internal standard and, after centrifugation at 5000 rpm/10 min, 1 mL of supernatant was reacted with sulfamic acid and triethyloxonium tetrafluoroborate to convert NO₃⁻ to EtONO₂ which was sampled in the headspace before GC-MS analysis.

113 2.4. GC-MS operative conditions

114 All measurements were performed on a Hewlett-Packard 5973 GC-MS system in EI mode.
115 The inlet was held at 120 °C and the headspace injection was performed with a 10:1 split
116 ratio. The use of a narrow liner (I.D. 1 mm) ensured a better chromatographic peak shape.
117 A constant on-column He flow rate was set at 1.2 mL/min and the gas sample was eluted on
118 the 30 °C isotherm with a DB-5.625 column (5%-phenyl-methylpolysiloxane: 30 m length
119 × 0.250 mm ID × 0.25 µm film). The transfer line was set at 200 °C. In these conditions,
120 the EtONO₂ derivative eluted at 2.4 min and could be detected in SIM mode at m/z 46

121 and 76 (50 ms dwell time). The derivative of the $^{15}\text{NO}_3^-$ internal standard showed the +1
122 shift and was therefore detected at m/z 47 and 77. The isotope ratio obtained by dividing
123 the peak areas extracted at m/z 46 over 47 was used for quantitation whereas the ratio at
124 m/z 76 over 77 was acquired as a qualifier. Automated integration was obtained with the
125 Agilent MassHunter quantitative software (Agile integrator). This method required minimal
126 instrumental maintenance, and allowed a sample throughput of 15 samples/hour.

127 **3. Results and discussion**

128 *3.1. Effect of drying on nitrate content in vegetables*

129 The experiments described in this paragraph aimed to demonstrate the stability of nitrate
130 during drying. We compared a soft, freeze-dry procedure with a more severe oven drying
131 process at 105 °C. A sample of fresh pre-washed spinach was purchased in Ottawa in April
132 2018 and frozen at -20 °C. The sample was homogenized with a blade food processor while
133 kept frozen. Aliquots of 2 g were portioned in 9 vials for nitrate extraction. Three of those
134 were kept at -20 °C, other three were placed in a static oven at 105 °C and the last three
135 were freeze-dried (Thermo Scientific, 5L ModulyoD). After 48 hours, the 9 samples were
136 analyzed for nitrate using the procedure described in paragraph 2.3. As reported in Table
137 1, the nitrate concentration of the fresh sample was 3.04 ± 0.06 mg/g. Consistently, the
138 nitrate content of freeze-dried and heat-dried samples were: 3.27 ± 0.19 mg/g and 3.29
139 ± 0.03 mg/g, respectively, when normalized to the fresh sample mass. The two drying
140 procedures did not impact the absolute amount of nitrate, demonstrating the stability of
141 this anion to conditions that may be encountered during food processing. Notably, the
142 drying procedure removed all water and volatile compounds present in the sample leaving

143 a residual mass equal to the 7% of the starting material. As a consequence, the vegetable
 144 powder was strongly enriched in nitrate, whose concentration on a dry weight basis exceeded
 145 40 mg/g (4% *w/w*). This tenfold enrichment of nitrate in the dried material does not find
 146 a match when compared to the effect of cooking on the nitrate content of vegetable. For
 147 example, reduction of the amount of nitrate was reported for boiled spinach (Prasad and
 148 Chetty, 2008) and kale (Kapusta-Duch et al., 2016). Conversely, nitrate enrichment, just up
 149 to a factor of three, was observed for fried spinach (Prasad and Chetty, 2008).

Table 1: Nitrate content on a spinach sample before and after drying

Spinach sample	Fresh mass (g)	Dried mass (g)	Mass loss (%)	mg/g NO ₃ ⁻ (on fresh)	mg/g NO ₃ ⁻ (on dry)
Fresh	1.5376	n/a	0%	3.00	n/a
Fresh	2.0299	n/a	0%	3.11	n/a
Fresh	2.3770	n/a	0%	3.02	n/a
Freeze-dry	1.7811	0.1256	92.9%	3.05	40.03
Freeze-dry	2.0798	0.1482	92.9%	3.37	43.18
Freeze-dry	2.5326	0.1822	92.8%	3.40	42.39
Dry at 105 °C	1.8188	0.1269	93.0%	3.26	43.06
Dry at 105 °C	2.1778	0.1516	93.0%	3.29	42.90
Dry at 105 °C	2.2292	0.1562	93.0%	3.32	43.01

150 3.2. Nitrate content in processed vegetables

151 Commercial vegetable products were screened for residual nitrate. Samples were acquired
 152 both locally in Ottawa’s stores and through on-line retailers. Two spinach powders, three
 153 kale powders, one beetroot powder, one beetroot crystal, two blends of grass powders (in-
 154 cluding Alfalfa, Barley, and wheat grass), two blends of vegetable powders, one broccoli
 155 powder, one Alfalfa grass powder, one Barley powder, and one beetroot juice were pur-
 156 chased. Before analysis all materials were homogenized: a sample aliquot of 40–60 g was
 157 transferred into a 250 mL Nalgene bottle which was shaken for 20 min on an elliptical paint

158 mixer. The beetroot juice was manually mixed. For nitrate analysis, 0.2 g aliquots of pow-
159 der (0.5 mL beetroot juice) were prepared according to the procedure outlined in paragraph
160 2.3. All measurements were carried out in triplicate. As reported in Table 2, the nitrate
161 content of these products varied from 0.61 mg/g in the Barley grass powder to 22.82 mg/g
162 in a spinach powder. In five out of fourteen samples, the nitrate content exceed the percent
163 level on mass fraction. Table 2 shows a strong enrichment of nitrate into vegetable powders
164 for domestic use. In the case of spinach and kale, the nitrate content of their dried powders
165 was about tenfold higher than median values reported for the fresh vegetables (Santamaria,
166 2006; Bahadoran et al., 2016; Kapusta-Duch et al., 2016). This evidence is consistent with
167 the observations reported in paragraph 3.1 and confirm that industrial drying does not affect
168 the chemical integrity of nitrate.

169 3.3. Nitrate exposure

170 As shown in Table 2, the nitrate content of commercial vegetable powders can exceed the 1%
171 level on dry mass and their consumption is significant for the daily intake of dietary nitrate.
172 A single serving of 30 g spinach powder (22.82 mg/g NO_3^-) yields 685 mg nitrate which is
173 triple the ADI (Table 2). A 250 mL beetroot juice portion gives 426 mg nitrate which is
174 double the ADI. The single serving size recommended for other products is in the 5–10 g:
175 already within such small amounts, the ADI can be easily surpassed when 3–5 servings are
176 consumed daily. The fact that a diet rich in vegetables can serve to exceed the ADI for
177 nitrate is known (Hord et al., 2009). However, there is a general agreement that potential
178 risks of *N*-nitrosation following ingestion of fresh vegetables are lowered by the action of
179 antioxidants which can inhibit the formation of nitrosamine, providing a natural protection

Table 2: Nitrate content in processed vegetables for human consumption

Material	NO ₃ ⁻ in veggie products (mg/g) ^a	Serving size (g) ^b	Serving NO ₃ ⁻ (mg)	Serving to ADI (%) ^c	Vit. C (% DV) ^d
Spinach powder	22.82 ± 0.17	30	685	308%	159%
Kale powder	15.58 ± 0.27	5	78	35%	n/a
Beetroot powder	14.37 ± 0.07	10	144	65%	7%
Kale powder	12.95 ± 0.13	5	65	29%	62%
Spinach powder	12.06 ± 0.14	8	96	43%	0%
Beetroot crystals	7.82 ± 0.03	10 ^e	78	35%	4%
Kale powder	6.48 ± 0.07	7	45	20%	60%
Grass powder	5.19 ± 0.10	5	26	12%	4%
Mix vegetable powder	4.11 ± 0.06	7	29	13%	64%
Grass powder	3.13 ± 0.04	7	22	10%	3%
Broccoli powder	1.81 ± 0.02	10	18	8%	n/a
Beetroot juice	1.70 ± 0.01	250	426	192%	0%
Mix vegetable powder	1.22 ± 0.06	9	11	5%	n/a
Alfalfa grass powder	1.01 ± 0.01	6	6	3%	n/a
Barley grass powder	0.61 ± 0.03	3	2	1%	0%

^a Standard deviation was calculated from three replicate measurements

^b Specified on the nutrition facts label by the manufacturer. In supporting information, Table S1 reports all other nutritional information

^c Calculated as the percent ratio between amount of NO₃⁻ per serving and 222 mg which is the ADI for a 60 kg person (Alexander et al., 2008)

^d Percent daily value of vitamin C for serving as reported by the manufacturer

^e Recommended 2–4 times per day by manufacturer

180 to the gastrointestinal function (Bartsch et al., 1988; Weitzberg and Lundberg, 2013). Whilst
 181 fresh vegetables are rich in vitamin C and other antioxidants, such essential nutrients may
 182 be depleted in the dried vegetable powders (Kapusta-Duch et al., 2016). Vega-Gálvez et al.
 183 (Vega-Gálvez et al., 2009) demonstrated that when a red paper sample was dried at 90 °C, a
 184 98.2% loss of vitamin C occurred. Korus (Korus, 2011) showed that an air-dried kale sample
 185 lost around 50% of its vitamin C after a shelf period of 12 months. Notably, the vitamin C
 186 level claimed in the nutrition facts label for beetroot juice and one spinach powder in Table
 187 2 was 0% of the daily value (Table 2). Considering the high level of nitrate contained in
 188 such products, along with their reduced antioxidant potential, more research is needed to

189 evaluate possible *N*-nitrosation reactions when vegetable powders are consumed alone or in
190 combination with foods rich in proteins or other *N*-nitrosatable precursors ([Vermeer et al.,](#)
191 [1998](#)).

192 **4. Conclusion**

193 The nitrate anion is a component typically present in fresh vegetables. When a spinach
194 sample was dried at 105 °C for 48 hours in a static oven, no degradation of incurred nitrate
195 was observed. As a consequence, the dried material showed a tenfold nitrate enrichment
196 with respect to the fresh one, consistent with the water loss. In light of this observation,
197 we screened several vegetable powders and we discovered that in many cases their nitrate
198 content exceed the percent level (>10 mg/g). The consumption of 1–5 servings/day of such
199 products could significantly exceed the ADI for nitrate. Considering recent debates on the
200 role of nitrate in the human diet, we believe that information about nitrate content in such
201 vegetable products should be stated on the information label for giving to the consumer ap-
202 preciation for nitrate consumption in relation to ADI.

203 The major concern for a nitrate-rich diet is rooted in the endogenous formation of car-
204 cinogenic nitrosamines. Fresh vegetables, which are among the first contributors to dietary
205 nitrate, have been considered safe because their antioxidants act as inhibitors toward *N*-
206 nitrosation. However, dried vegetable powders do not match the antioxidant properties of
207 fresh vegetables, and if not properly rehydrated, they may contribute to localized, high-
208 nitrate concentration in the digestive system which could influence the reaction kinetics of a
209 number of processes. More investigations are needed to clarify the safety aspects of dietary
210 nitrate assumed through consumption of dried vegetables and beetroot juice with particu-

211 lar attention to the combination of such products with foods containing high quantities of
212 *N*-nitrosatable precursors.

213 **5. Conflict of interest**

214 There are no conflicts of interest.

215 **6. Acknowledgment**

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217 the manuscript.

218 **References**

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