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Quantitative and Comparative Contents of Nitrate and Nitrite in *Beta vulgaris* L. by Reversed-Phase High-Performance Liquid Chromatography-Fluorescence

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Abstract The consumption of beetroot, a food rich in nitrate, has been recommended to enhance blood perfusion, restore endothelial function, and improve exercise performance. These properties may be explained by the possible effect of nitrate present in beetroot in stimulating the endogenous synthesis of nitric oxide, a potent vasodilator. However, there is limited evidence giving detailed information about how nitrate contents from beetroot used in studies have been analyzed. The purpose of this study was to evaluate nitrate and nitrite contents of beetroot from different regions of Brazil and from the USA. Nitrate and nitrite contents were quantified by using a reversed-phase high-performance liquid chromatography system with fluorescence detection. Beetroots from the USA showed the highest nitrate (31.2±0.010 mmol.L^{-1}) and nitrite (0.45±0.005 mmol.L^{-1}) contents when compared to beetroots from Brazil. Rio de Janeiro was the region that showed the highest nitrate content (17.1±0.020 mmol.L^{-1}), while Rio Grande do Norte presented the highest nitrite content (0.13±0.010 mmol.L^{-1}). The reversed-phase high-performance liquid chromatography-fluorescence method may be applied for the quantification of nitrate and nitrite contents in beetroot samples. The results of the present study demonstrate that beetroots may vary in their nitrate and nitrite contents. This data represents a useful tool to encourage researchers to analyze the nitrate and nitrite contents in beetroots utilized in interventional clinical studies.

Keywords Food composition · Beetroot · High-performance liquid chromatography · Nitrate · Nitrite

Introduction

Beetroots belonging to the *Beta vulgaris* L. species are considered a source of fibers, antioxidants, minerals, and carbohydrates. In addition, the vegetable is also considered a dietary NO_3^- source (Pennington 1998; Santamaría 2006; Tamme et al. 2006). The NO_3^- content in this food matrix has been proposed to enhance blood perfusion (Webb et al. 2008; Kapil et al. 2010), restore endothelial function (Stokes et al. 2009), and improve exercise performance (Vanhatalo et al. 2011; Murphy et al. 2012). These properties may be explained by the possible effect of NO_3^- present in beetroot juice in stimulating the endogenous synthesis of nitric oxide (NO).

Different NO_3^- contents of beetroot juice have been used in order to stimulate NO production, ranging from 5.2 to 22.5 mmol. Vanhatalo et al. (2010) observed a significant increase in NO production (evaluated indirectly by plasma nitrite [NO_2^-]) after the consumption of 5.2 mmol of NO_3^- (500 mL of beetroot juice) by healthy subjects. Webb et al. (2008) and Lansley et al. (2011) observed an increase in NO production after the ingestion of 22.5 and 6.2 mmol of NO_3^- (500 mL of beetroot juice). Kapil et al. (2010) and Wylie et al. (2013) observed an increase in NO production after the ingestion of 5.5 mmol (250 mL of beetroot juice) and 16.8 mmol of NO_3^- (280 mL of beetroot juice), respectively. Cermak et al. (2012) observed an increase in NO production after the ingestion of 8.7 mmol of NO_3^- (140 mL of beetroot juice), respectively.

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However, only two of the aforementioned studies (Vanhatalo et al. 2010; Bailey et al. 2009) detailed information about how NO_3^- and NO_2^- contents from beetroot juice used in their interventions were analyzed. Analyzing NO_3^- and NO_2^- contents of different beetroot juices could be an important methodological strategy in order to guide researchers, because the physiological effects of beetroot may be directly related to NO_3^- content and not necessarily to the volume of beetroot juice consumed. Furthermore, there is a tendency to assume that the information on NO_3^- content given by commercial juice manufacturers (Webb et al. 2008; Kapil et al. 2010; Murphy et al. 2012; Wylie et al. 2013) is exact. However, juice manufacturer information on NO_3^- content may not necessarily reflect the real NO_3^- content of natural beetroot juice as given to volunteers during studies.

A variety of analytical methods to determine NO_3^- and NO_2^- has already been performed for the analysis of these anions in biological samples, food, plants, water, agricultural products, and other matrices. These techniques include the Griess colorimetric assay, electrochemical detection, chemiluminescence, and capillary electrophoresis (Jimidar et al. 1995; Cheng and Tsang 1998; Siu and Henshall 1998, Tsikas 2007). However, these methods present disadvantages (Jobgen et al. 2007).

Since NO_3^- content may also vary according to agricultural management, landscape, and genetic factors (Tamme et al. 2006)—and there is a lack of available information about NO_3^- content in natural beetroot juice from different areas—the present study was conducted in order to evaluate the NO_3^- and NO_2^- contents in red beetroot *in natura* from different Brazilian regions by reversed-phase (RP) high-performance liquid chromatography (HPLC) method with fluorescence detection. The NO_3^- and NO_2^- contents in beetroot acquired in the USA were also evaluated.

Material and Methods

Instruments and Chemicals

High-Performance Liquid Chromatography System

The HPLC system consisted of a quaternary pump model LC-20AD (Shimadzu®, Tokyo, Japan), auto sampler model SIL-10AF (Shimadzu®, Tokyo, Japan), column oven model CTO-20A (Shimadzu®, Tokyo, Japan), and fluorescence detector model RF-20A (Shimadzu®, Tokyo, Japan). A reversed-phase C18 column (3.5 μm, 100×4.6 mm, I.D., Kromasil®, Bohus, Sweden) guarded by a reversed-phase C18 guard column (5 μm, 50×4.6 mm, I.D., Nucleosil®, Pennsylvania, USA) was used for separation of the analytical compounds.

Chemicals

All chemicals used in the present study were of analytical grade. Monobasic and dibasic sodium phosphate and HPLC grade methanol was purchased from Tedia (Tedia Company, INC., OH, USA). Purified and deionized water was obtained from a Milli-Q water purification system (Millipore, Molsheim, France) and was used to prepare the mobile phase solution, reagents, samples, and standards. Nitrate reductase (*Aspergillus* species, EC 1.6.6.2) and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Roche (Roche Diagnostics, Mannheim, Germany). Sodium hydroxide (NaOH) was obtained from Reagen (Ultrapure Chemicals of Brazil, Ltda., Rio de Janeiro, Brazil). Sodium NO_3^- and NO_2^- standards, 2.3-diaminonaphthalene (DAN), and flavin adenine dinucleotide (FAD) were obtained from Fluka (Sigma-Aldrich, São Paulo, Brazil). Hydrochloric acid (HCL) was obtained from Vetec (Vetec Quimica Fina, Ltda., Rio de Janeiro, Brazil).

Sample Preparation for Analysis

Three fresh red beetroots (*B. vulgaris* L.) were acquired from five different markets (3 samples of each region × 5 markets; $n=15$) located in the following regions of Brazil: (1) Northeast (State of Rio Grande do Norte: 5°47′07.3″S, 35°12′14.6″"W and State of Bahia: 11°51′16.5″S, 40°50′19.3″W), (2) South (State of Santa Catarina: 27°35′45.8″S, 48°32′51.9″W), and (3) Southeast (State of Rio de Janeiro: 22°51′55.8″S, 43°13′34.1″W and State of Minas Gerais: 17°43′56.6″S, 44°35′00.7″W). Furthermore, three red beetroots of the same species were acquired in a New York supermarket (USA—40°47′23.5″S, 73°57′13.0″W). Beetroots were produced in vegetable gardens near the region in order to supply the markets with fresh vegetables. In New York, the beetroots were cultivated in an urban farm and sold in a fresh vegetable market. The beetroots had no cracks, stains, bruises, and wet areas, which indicate deterioration. The vegetables were packed in black sealed plastic bags and stored at 5 °C for 2 days before being transported. These procedures conducted during vegetable purchase and storage aimed to avoid damage and cuts that could expose the inner tissues to microbial contamination, higher deterioration rates, and metabolism acceleration (Watada et al. 1996). The beetroots were weighed, cleaned with deionized water, and processed by using a centrifuge food processor (EC 700, Black & Decker, São Paulo, Brazil) without adding any water. A mass of 0.350 kg of beetroot was sufficient to produce 100 mL of juice. The juice was diluted in 1:2000 and 1:100 ratios in order to analyze NO_3^- and NO_2^-, respectively. After dilution, each sample was filtered using a 0.22-μm syringe filter (Syringe-driven Filter Unit, Millex®, Merck Millipore Ltd., Carrigtwohill, Ireland). NO_3^- from the sample or standard (containing NO_3^-) was enzymatically

converted to NO_2^- by an enzyme, NO_3^- reductase, in the presence of NADPH/FAD cofactors. Briefly, the HPLC-fluorescence method is not capable of directly analyzing NO_3^-, thus, first, it is necessary to convert it to NO_2^-. One hundred microliters of the sample (diluted and filtered) or NO_3^- standard solution (0–2 μM) were treated by adding 10 μL of 1 U/ml NO_3^- reductase in the presence of 5 μL of 120 μM NADPH and 5 μL of 2 μM FAD. The conversion was carried out for 1 h at 24 °C in a water bath (Nova Instruments, São Paulo, Brazil). Ten microliters of 316 μM DAN in 0.62 M HCl, that reacts rapidly with NO_2^- under acid conditions to form 2.3-naphthotriazole (NAT), a highly compound fluorescent, were added to the resulting NO_2^-. After 10 min, NaOH was added to stabilize the fluorescent compound to be analyzed by an HPLC system equipped with a SIL-10AF auto sampler.

RP-HPLC-Fluorescence Analysis

The mobile phase solution, which consisted of 15 mM sodium phosphate buffer (pH 7.5) and methanol (50:50 ; *v*/*v*), was allowed to pass through the HPLC column for 5 min until a stable baseline signal was obtained. The isocratic flow rate was 1.1 mL.min^{-1}. When injections of the NO_3^- and NO_2^- standard solutions (five each calibration curves with six points concentration) gave reproducible retention times and a peak area, each sample solution was then injected for analysis. The amount in microliters of solvents until reproducible retention times was of 38.5 mL for NO_3^- and NO_2^- standard solution. The fluorescence was monitored with excitation at 375 nm and emission at 415 nm. Retention times were approximately 3 min for NO_3^- and NO_2^-. The sample peaks were identified by comparison to the respective standard peaks. The amounts of NO_3^- and NO_2^- in each sample were calculated from the peak areas by using linear regression equations of the NO_3^- and NO_2^- standard curves. The injection volume was 15 μL. At the end of the analyses, the RP-HPLC column was cleaned by running a 100 % water and 100 % methanol solution for 40 min for each solvent.

Statistical Analyses

A one-way analysis of variance (ANOVA) was used to identify differences in NO_3^- and NO_2^- contents between beetroots of different regions of Brazil and the USA. When a significant F was found, additional post hoc tests with Bonferroni adjustments were performed. Statistical significance was set at the 0.05 level of confidence. All analyses were performed using a commercially available statistical package (IBM SPSS® Statistics version 20 for Windows, Chicago, IL, USA). The results regarding the NO_3^- and NO_2^- contents of all analyzed beetroot were converted from μmol.L^{-1} to mmol.L^{-1} of NO_3^- and NO_2^- in beetroot juice in order to conduct comparisons with other studies published in the literature.

Results

Linearity

Figure 1 shows a very good linearity of NO_2^- and NO_3^- determinations within the range of 0.125 to 4 μmol.L^{-1}. Linearities were obtained over the tested concentration ranges from 0.125 to 4 μM for both NO_2^- and NO_3^-. The linear regression equations were calculated as $y=2E+06x+119{,}441$ ($R^2=0.9997$) for the NO_2^- and $y=2E+06x+111{,}643$ ($R^2=0.9999$) for NO_3^- standard analysis.

Limit of Detection

The limit of detection for NO_2^- and NO_3^- was of 1.0×10^5 μmol.L^{-1}, based on a signal-to-noise ratio of 4 when using the RF-20A fluorescence detector. The limit of detection of the present HPLC method for NO_2^- and NO_3^- analyses is much higher than that of the Griess colorimetric assay (limit of detection of 2 μmol.L^{-1}) (Jobgen et al. 2007). The limits of detection of the chemiluminescence method for NO_2^- and NO_3^- have been reported to be approximately 1.0×10^6 μmol.L^{-1} and 8.0×10^5 μmol.L^{-1}, respectively (Tsikas 2007). Thus, this assay is less sensitive than the RP-HPLC-fluorescence method for NO_3^- analysis.

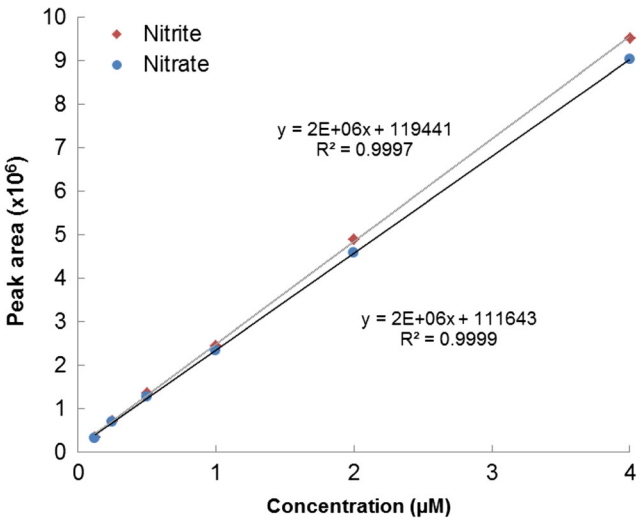

Fig. 1 NO_2^- and NO_3^- standard curves. The linear regression equations for the NO_2^- and NO_3^- standard curves were calculated as $y=2E+06x+119{,}441$ ($R^2=0.9997$) and $y=2E+06x+111{,}643$ ($R^2=0.9999$), respectively; where y is the value of peak area and x is the value of the various concentrations of the standard solutions using the HPLC-fluorescence method

Reproducibility

In this paper, only intra-day and inter-day coefficient of variation (CV, %) were considered to assess the reproducibility of the obtained values, which were calculated by the following formula: CV=[(mean of SD/mean of replicates measurements)*100]. The CV (n=6 replicates) for NO_3^- and NO_2^- were 0.67 and 0.54 % for intra-assays and 1.47 and 1.33 % for inter-assays, respectively. Repeated trails all obtained CV values of less than 1.5 %, pointing out high degrees of reproducibility.

NO_3^- and NO_2^- Contents in Beetroots

Figure 2 shows a representative chromatogram of NO_3^- and NO_2^- contents in beetroot juice analyzed by RP-HPLC-fluorescence.

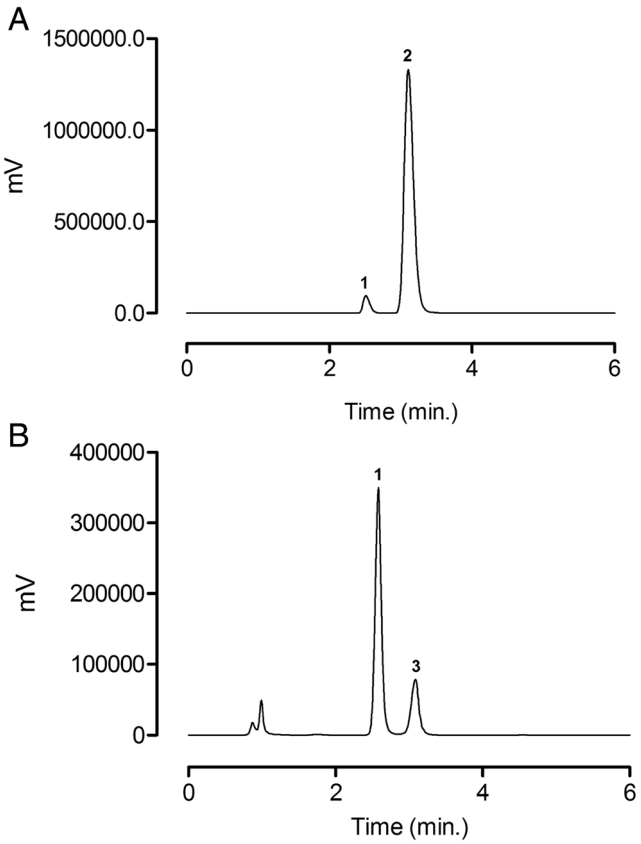

Fig. 2 Representative HPLC chromatogram of NO_3^- and NO_2^- analyses in beetroot juices. **a** Corresponds to a chromatogram analysis of NO_3^- in beetroot juices. NO_3^- was converted to NO_2^- by NO_3^- reductase enzyme. Then, NO_2^- reacted with 2.3-diaminonaphthalene (DAN) to yield 2.3-naphthotriazole (NAT) under acidic conditions. **b** Corresponds to a chromatogram analysis of the NO_2^- in beetroot juice. NO_2^- reacted with 2.3-diaminonaphthalene (DAN) to yield 2.3-naphthotriazole (NAT) under acidic conditions. The NAT (NO_2^--DAN) derivative was separated by RP-HPLC-fluorescence method: DAN (1); NO_3^- (2); NO_2^- (3)

The NO_3^- content of beetroots from distinct geographical regions in Brazil and from Northern USA are compared in Table 1. Among the Brazilian beetroots, the beetroots from Rio de Janeiro showed the highest NO_3^- content. Beetroots from the Minas Gerais and Bahia states showed the lowest NO_3^- contents. Beetroot from Northern USA (31.2± 0.010 mmol.L^{-1}) showed the highest NO_3^- content when compared to Brazilian beetroots. The NO_2^- contents of beetroots from distinct geographical regions were also estimated in Table 2. The NO_2^- contents were higher in beetroots from the Rio Grande do Norte and Minas Gerais states. The beetroots from two locations in southeastern Brazil, Rio de Janeiro and Santa Catarina states, showed the lowest NO_2^- contents among the Brazilian beetroots. The beetroots from Northern USA also showed the highest NO_2^- content (0.45± 0.005 mmol.L^{-1}) when compared to Brazilian beetroots.

Discussion

The beetroot belonging to the *B. vulgaris* L. species is also considered a dietary source of NO_3^- (Santamaría 2006; Tamme et al. 2006). The NO_3^- present in this food matrix has been associated with improved cardiovascular function (Hobbs et al. 2012; Lidder and Webb 2013). The present study was designed with the purpose of evaluating the NO_3^- and NO_2^- contents in Brazilian and US red beetroots by the RP-HPLC method with fluorescence detection.

The main finding of the present study was that beetroots from Rio de Janeiro showed significantly high NO_3^- content when compared to beetroots from Rio Grande do Norte, Santa Catarina, Minas Gerais, and Bahia. Beetroots from Rio Grande do Norte showed significantly high NO_2^- content when compared to beetroots from Minas Gerais, Bahia, Rio

Table 1 Nitrate (NO_3^-) contents of beetroots acquired from different regions of Brazil and from the USA

Region	mmol.L^{-1}
Northeastern Brazil	
Bahia	5.74±0.033 e
Rio Grande do Norte	12.54±0.150 c
Southeastern Brazil	
Minas Gerais	6.46±0.057 e
Rio de Janeiro	17.1±0.020 b
Southern Brazil	
Santa Catarina	10.0±0.056 d
Northern USA	
New York	31.2±0.010 a

The values are presented as means±SD. Different letters denote statistical significance between the samples at P<0.05. Each juice sample (aliquot) was analyzed in quintuplicate

Table 2 Nitrite (NO_2^-) contents of beetroots acquired from different regions of Brazil and from the USA

Region	mmol.L^{-1}
Northeastern Brazil	
Bahia	0.06±0.006 d
Rio Grande do Norte	0.13±0.010 b
Southeastern Brazil	
Minas Gerais	0.11±0.006 c
Rio de Janeiro	0.04±0.001 e
Southern Brazil	
Santa Catarina	0.04±0.006 e
Northern USA	
New York	0.45±0.005 a

The values are presented as means±SD. Different letters denote statistical significance between the samples at $P<0.05$

de Janeiro, and Santa Catarina. Beetroots from the USA showed higher NO_3^- and NO_2^- contents when compared to all beetroots acquired in Brazil.

Several factors affect the uptake and accumulation of NO_3^- contents in vegetables (Habermeyer et al. 2015). Agricultural (type of cultivation, fertilization, use of herbicides, and availability of other nutrients) and environmental (atmospheric humidity, temperature, exposure to sunlight, and light intensity) factors are the factors that most affect NO_3^- content in beetroots (Santamaría 2006; Habermeyer et al. 2015). Originally from European and North African regions, the beetroot is a vegetable typical of temperate climates, which adapts well in temperatures between 10 and 20 °C (Tullio et al. 2013). In the present study, the differences in NO_3^- content of beetroots from Rio de Janeiro compared with other Brazilian regions may be due to their cultivation during winter, when the climate is similar to that of the USA and Europe. Beetroot production in Rio de Janeiro in the winter period coincides with the greater availability of this vegetable in this state. With high temperatures (>30 °C), the state of Bahia has a tropical climate that is totally unsuitable for the cultivation of beetroot, which may be the reason that the beetroot from Bahia presented the lowest NO_3^- content compared to the other Brazilian beetroots. Although beetroots from Rio Grande do Norte presented the highest NO_2^- content when compared to the other Brazilian regions, the values of this anion were low in all the analyzed Brazilian beetroots. These results were already expected, since the beetroot is a vegetable that has a non-significant NO_2^- content (Pennington 1998; Tamme et al. 2006).

Besides the fact that the predominantly temperate climate in the USA is conducive to better beetroot development when compared to subtropical Brazil, a possible explanation for the differences in the NO_3^- and NO_2^- contents of beetroots from the USA and from different regions of Brazil may be due to agricultural factors. The use of nitrogen-based fertilizers also affects beetroot NO_3^- and NO_2^- contents (Pussemier et al. 2006). Thus, it is plausible that a more intensive usage of such fertilizers may explain the higher NO_3^- and NO_2^- content from the USA when compared to beetroots from Brazil.

Recently, several intervention studies have been conducted using different amounts of beetroot (in juice form) for investigating the contribution of NO_3^- in NO synthesis (Webb et al. 2008; Bailey et al. 2009; Stokes et al. 2009; Kapil et al. 2010; Vanhatalo et al. 2011; Murphy et al. 2012; Wylie et al. 2013). However, most of these studies did not assess the NO_3^- and NO_2^- contents of the administered beetroot juices. The authors reported only the values based on information previously established by beetroot juice manufacturers. Fulford et al. (2013) administered 500 mL of beetroot juice supplied by James White Drinks Company Ltd. (Ipswich, UK). The authors reported that this amount of juice contained 5.1 mmol (or 10.2 mmol.L^{-1}) of NO_3^-. However, no information about how this content was evaluated was provided. Many other studies also did not provide this information (Webb et al. 2008; Kapil et al. 2010; Vanhatalo et al. 2010; Lansley et al. 2011; Cermak et al. 2012; Hobbs et al. 2012; Wylie et al. 2013).

The results of the present study demonstrate that the NO_3^- contents in 1 L of juice of beetroot acquired in Rio de Janeiro and the USA were of 17.1±0.020 mmol and 31.2±0.010 mmol, respectively. The NO_3^- contents in beetroot juice differ from those described by Webb et al. (2008), Bailey et al. (2009), Vanhatalo et al. (2010), and Kelly et al. (2013), which reported values of 45.0, 11.2, 10.4, and 68.5 mmol.L^{-1}, respectively. Kapil et al. (2010) also administered 250 mL of beetroot juice to nine healthy subjects, reporting that this amount contained 5.6 mmol (or 22.4 mmol.L^{-1}) of NO_3^-, and observed a significant reduction in systolic blood pressure. On the other hand, Gilchrist et al. (2013) also administered 250 mL of beetroot juice to 27 patients with type 2 diabetes mellitus, reporting a content of 7.5 mmol (or 30 mmol.L^{-1}) of NO_3^-, and did not observe any reduction in blood pressure. The information provided by studies about NO_3^- content may not correspond to the actual content of this anion present in beetroot juices provided to volunteers. The possibility of such a discrepancy may be the reason why some studies observed different physiological effects following beetroot juice administration.

Furthermore, the discrepancies among the NO_3^- contents of the beetroot juices of the aforementioned studies show that it is crucial to precisely evaluate NO_3^- and NO_2^- contents in these tubers using the appropriate analytical techniques. Chemiluminescence analyses and Griess colorimetric assays are the most commonly used methods, but the chemiluminescence analysis shows certain disadvantages, such as bulky apparatus, low specificity and sensitivity, and inability to directly detect NO_3^-. Although the Griess reaction is simple, this sample amounts low sensitivity and specificity (Jobgen et al. 2007; Hetrick and Schoenfisch 2009).

Conclusion

In conclusion, although beetroot juice has been consumed for its high NO_3^- content, the results of the present study demonstrate that beetroots from different regions of Brazil may vary in their NO_3^- and NO_2^- contents. Therefore, it is important that studies investigating beetroot juice analyze NO_3^- content by using the appropriate analytical techniques. The RP-HPLC-fluorescence method is recommended to determine NO_3^- and NO_2^- in beetroots. The data from the present study represents a useful tool to encourage researchers to analyze the NO_3^- and NO_2^- contents in food that is administered with the intention of observing physiological and biochemical effects of these compounds on human health.

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Compliance with Ethical Standards This article does not report any studies with human or animal subjects.

Conflicts of Interest Diego Baião declares that he has no conflict of interest. Carlos Conte-Junior declares that he has no conflict of interest. Vânia Paschoalin declares that he has no conflict of interest. Thiago Alvares declares that he has no conflict of interest.

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