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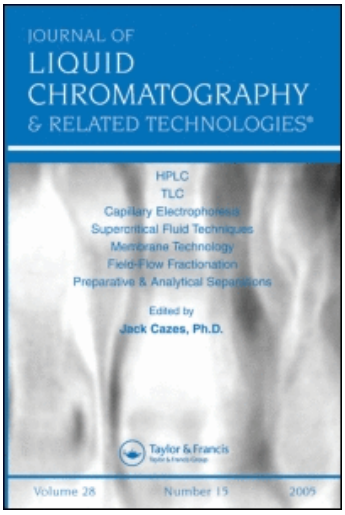

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Edgar Pinto[a]; Catarina Petisca[a]; Luís F. Amaro[ab]; Olívia Pinho[ab]; Isabel M. P. L. V. O. Ferreira[a]
[a] REQUIMTE-Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal [b] Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto, Portugal

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INFLUENCE OF DIFFERENT EXTRACTION CONDITIONS AND SAMPLE PRETREATMENTS ON QUANTIFICATION OF NITRATE AND NITRITE IN SPINACH AND LETTUCE

Edgar Pinto,[1] Catarina Petisca,[1] Luís F. Amaro,[1,2] Olívia Pinho,[1,2] and Isabel M. P. L. V. O. Ferreira[1]

[1]*REQUIMTE-Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal*
[2]*Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto, Portugal*

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□ *Different sample pretreatment and extraction techniques are often used for analysis of nitrates and nitrites, however, the effects of these variables have not been properly examined. Comparative investigations were carried out with the objective of finding the most suitable conditions for quantification of nitrate and nitrite in spinach and lettuce. A rapid and cost effective RP-HPLC/UV method was validated and used to select the most appropriate extraction procedure to eliminate chromatographic interferences and to evaluate the influence of different sample pretreatments on the accuracy and reproducibility of the results obtained. Similar nitrate concentrations were obtained for fresh and two weeks frozen samples. Freeze drying and oven drying pretreatment of the spinach and lettuce material was inappropriate. No nitrite was detected in either fresh, freeze dried, oven dried, and frozen spinach or lettuce.*

Keywords lettuce, nitrate, nitrite, RP-HPLC/UV, spinach

INTRODUCTION

Vegetables are an outstanding source of vitamins, minerals, and biologically active compounds, playing an important role in human nutrition. However, the health problems posed by nitrate and nitrite in vegetables have been a focus of attention in many countries. Vegetables tend to concentrate nitrate ions, thus, they are a major source of human exposure to these compounds, especially if grown using a high application of N fertilisers. Nitrate concentrations vary significantly, ranging from 1 to 10,000 mg kg^{-1} fresh weight, while nitrite levels in fresh vegetables are

Address correspondence to Isabel M. P. L. V. O. Ferreira, REQUIMTE-Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha 164, 4099-030 Porto, Portugal. E-mail: isabel.ferreira@ff.up.pt

low (<2 mg kg^{-1}).[1] Nitrite levels in vegetables may increase during post harvest storage by the action of indigenous bacteria and/or the presence of nitrate reductase,[2] especially when they are left at room temperature or higher. Cultivar and harvest date can affect the nitrate and nitrite levels of selected vegetables.[3]

Nitrate has a low level of acute toxicity but may be transformed into nitrite, which may lead to the formation of carcinogenic nitrosamines. High dietary nitrate and nitrite intake may increase the risk of gastrointestinal cancers due to the in vivo formation of carcinogenic N-nitroso compounds.[4] The stomach is most at risk from endogenous N-nitroso compound synthesis since stomach acid catalyses nitrosation reactions. Moreover, excessive intake of nitrite and nitrate in the diet may cause toxic effects since methaemoglobinaemia is produced by oxidation of haemoglobin by nitrite, and infants under 6 months of age are particularly susceptible.[5] Nitrate contamination in vegetables occurs when crops absorb more than they require for their sustainable growth. Spinach, lettuce, and other vegetables possess the tendency to accumulate nitrates.[6] Consequently, the European Commission/EC) established maximum levels of nitrate in lettuce and spinach.[7] The vegetable producers should gradually modify their farming methods by applying the codes of Good Agricultural Practice (GAP) recommended at national levels, so as to comply with the maximum levels to reduce nitrate levels.

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A variety of analytical methods for the determination of nitrate and nitrite have been developed and applied to analysis of food, water, plants, and other matrices. Nitrite, and nitrate after reduction to nitrite, are routinely measured in food by spectrophotometric methods based upon the ability of nitrite to convert aromatic amines into diazonium ions, which, in turn, are coupled to another aromatic compound in order to produce an azo dye (the Griess-Romijn reaction).[8] The most common arrangement utilises sulphanilamide and N-(1-naphthyl)ethylenediamine as the target amine and coupler, respectively, with the product of the reaction detected at 540 nm. A variety of reducing agents have been investigated to facilitate this conversion and include amalgamated cadmium,[9] copperised cadmium,[10] and zinc,[11] and more recently, photo-induced reduction,[12] Other current methods for the determination of nitrite and nitrate rely on segmented flow or flow injection analysis variants of the traditional colourimetric Griess diazotisation procedure.[13–17] These methods are traditionally used to determine nitrite and nitrate in food, however, a lack of high sensitivity for the detection of trace levels of the analytes can cause unreliable results due to sample matrix interferences.

Alternative methods for nitrate and nitrite determination in foodstuffs have also been developed, including spectroscopic determination after enzymatic reduction,[18] polarography,[19] and capillary

electrophoresis.[20,21] Ion chromatographic methods have also been widely studied for the separation of nitrite and nitrate and other ions in several matrices.[22–25] Ion pair HPLC methods offer, with respect to ion chromatography, advantages of relatively lower cost in instrumentation and columns.[26,27]

Nitrate and nitrite can be unstable and appropriate sampling methods and extraction procedures must be chosen to obtain reliable results. Extraction into hot water (or borax) is the most usual process.[4,21,26] It is recommended that samples are analysed as soon as possible after collection. Howerer, this may be impractical when the sampling is done at a distance from the laboratory or a high number of samples is analysed. It may be necessary to store the samples before analysis. Freeze, freeze dried, or oven dried are the usual procedures described in literature for storage of vegetables before nitrate and nitrite analyses,[28–31] but there is no information related with reliability of these processes. The objectives of this study were to evaluate the influence of these sample pretreatments on the accuracy and reproducibility of results and chose the most appropriate extraction procedure to eliminate chromatographic interferences and preserve the chromatographic column.

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EXPERIMENTAL

Chemicals and Samples

All reagents used were of analytical grade purity. Solvents for HPLC were filtered trough 0.22 μm NL 17 filters and degassed under vacuum for at least 15 min before use. Sodium nitrate, sodium nitrite, and n-octylamine were supplied by Sigma Chemicals Co. (St Louis, MO, USA), methanol (Licrosolv) and activated charcoal was from Merck (Darmstradt, Germany). Standard solutions of sodium nitrite (1000 mg/L) and sodium nitrate (1000 mg/L) were prepared from $NaNO_3$ and $NaNO_2$ previously dried in an oven (100°C during 1 hour). More diluted standard solutions, used in the calibration curves, were obtained from the concentrated solutions by dilution. The solutions were treated with some chloroform drops to prevent the development of microorganisms and were stored in a refrigerator.

Instrumentation

The chromatographic analysis was carried out in an analytical HPLC unit (Gilson, France) equipped with a type 305 pump and a type 7125 Rheodyne Injector with a 20 μL loop, a Gilson 118 variable long wave ultra

violet detector ($\lambda = 220$ nm) and a Gilson 712 HPLC System Controlller Software. The chromatographic separation was achieved using a ACE C18, 5 μm chromatographic column and isocratic elution with 0.01 M n-octylamine and 20% methanol to pH 6.6. The flow rate was 0.5 mL/min.

Fungilab Ultrasonic cleaner and a METROHM 632 pH-Meter were used for eluent preparation. A vortex Heidolph REAX 2000 was used for sample preparation.

Prior to analysis, the ion interaction reagent solution was allowed to pass through the HPLC column until a stable baseline signal was obtained. Generally, a stabilization time of 30 minutes was required before analysis, and then reproducible retention times were observed throughout the working day (8–12) hours of analysis. At the end of the working day, the HPLC column was regenerated by passing 3:7 water-methanol overnight at a flow rate of 0.2 mL min^{-1}.

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Sample Collection

Fresh leafy vegetables, i.e., spinach (*Tetragonia tetragonioides*) and lettuce (*Lactuca sativa*) were collected from different fields.

Sample Preparation

Non-edible parts of each sample were removed and vegetables were frozen at − 20°C during 6 hours. Then, samples were cut, homogenized, and divided in three lots. The first lot was analysed fresh (less then 24 hours after collection, codified as S or L, for spinach or lettuce, respectively), the second lot was frozen during two weeks (codified as, SF or LF, respectively, for spinach or lettuce), and the third lot was freeze dried and sifted through a pore less than 500 μm^6 (these samples were codified as SFd or LFd, for spinach or lettuce, respectively). Additionally, fresh vegetable leafs were taken and dried in a force air oven (Model WTC Binder 78532) to 70°C for 48 h as described by Castro et al.[31] The dried leaves were then ground in a mill and sifted through a pore less than 500 μm and codified as SO and LO, for spinach or lettuce, respectively.

Dry matter of fresh, frozen, freeze dried and oven dried samples was evaluated using an oven from Scaltec Instruments (Goettingen, Germany), at 100°C.

The use of an effective material to remove interferences from vegetable matrices was tested; activated charcoal may meet this demand owing to its cheapness and strong adsorption character. The homogenized sample, usually, 0.250 g for fresh and frozen samples and 0.025 g for freeze dried and over dried samples (however, amounts ten times higher could be used

for evaluation quantification limits) was weighed, put into a 100 mL volumetric flask amongst equal amount of activated charcoal, and then 50 mL deionised water was added. Similar procedure was performed without addition of activated charcoal. The flasks were heated for 20 min at 80°C, shaken, allowed to cool, and then diluted to a final volume of 100 mL with deionised water. After filtration through a 0.45 mm syringe filter, the filtrate was analysed for nitrate and nitrite by high performance liquid chromatography/UV.

Method Validation

Each batch consisted of replicate analyses of blanks (limit of detection), standard solutions (sensitivity and linear range) and both spiked and unspiked samples (recovery and precision). Linearity was addressed by preparing five standard solutions of sodium nitrite and sodium nitrate ranging between 0.05 to 20.0 mg L^{-1}. A linear regression analysis of analyte concentration vs peak response was performed. The detection limits were calculated as the concentration corresponding to three times the background noise of the blank. Intra-day (running 3 times on the same day), and interday tests (running 6 times within successive 7 days with at least 24 h as intervals) were conducted. The reproducibility precision values were characterized by the relative standard deviation (RSD, %).

For recovery studies a series of concentrations of standard solutions containing nitrate and nitrite were spiked into organic spinach and lettuce samples. Each concentration spiked was analyzed in triplicate, including a blank test to evaluate the average recoveries.

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Statistical Design

Data were subjected to ANOVA treatments and the Duncan test used to discriminate among means at $p < 0.05$. To ensure data were of normal distribution, standardized skewness and standardized kurtosis values were checked.

RESULTS AND DISCUSSION

Performance of HPLC Method and Selection of Extraction Conditions

In this study, a simple, efficient, and accurate HPLC method mainly derived from the procedures of Cheng and Tsang[25] was adapted for the determination of nitrate and nitrite in spinach and lettuce. Isocratic

elution with a mobile phase containing 0.01 M *n*-octylamine/20% methanol, pH 6.6, enables nitrite and nitrate ion pair chromatographic separation. Under the experimental conditions described, the retention time of the target analytes was very reproducible. The retention times of nitrite and nitrate were 11.28 ± 0.03 min and 14.67 ± 0.07 min, respectively. The total analytical time of the method for one sample analysis was within 15 min.

Linearity was obtained over the tested concentration range of 0.05–20 mg L^{-1} of nitrate and nitrite, respectively. The linear regression equations of nitrate and nitrite standard curves were calculated as $y = 19718x + 1225.3$ and $y = 26653x - 3765.2$, respectively. The correlation coefficients were both greater than 0.999, which indicated very good linearity. The calibration curves were used to calculate concentration of nitrate and nitrite in spinach and lettuce samples and finally reported as mg kg^{-1}.

The detection limit of nitrate and nitrite, defined as a signal-to-noise ratio of 3, was 0.02 mg L^{-1}. The method showed good sensitivity and can detect trace levels of nitrate and nitrite (<2 mg kg^{-1}).

Reproducibility of the measurements was evaluated by intra-day and inter-day analysis calculated from the results of repeated determinations of 5 mg L^{-1} standard solution of nitrate and nitrite and illustrated by the relative standard deviation (RSD, %), as shown in Table 1. RSD values were in general less than 3%.

The HPLC procedure was applied to the analyses of fresh spinach and lettuce samples and the sample amount used was chosen to fall within the standard calibration curve range.

Two extraction techniques were assessed, *viz* with and without addition of activated charcoal using fresh and spiked vegetable samples. Activated charcoal was efficient to remove interferences from vegetable matrices as can be observed in Figs. 1a and b. Several chromatographic peaks were observed in spinach samples extracted without addition of activated charcoal (Fig. 1a), including one peak with retention time near to that of nitrite. These interfering peaks were almost removed using activated

TABLE 1 Reproducibility of Inter-day and Intra-day Analysis

	Precision (RSD %)[c]			
	Retention time (min)		Concentration (5 mg L^{-1})	
	Run-to-run[a]	Day-to-day[b]	Run-to-run[a]	Day-to-day[b]
Nitrite	0.05	2.70	1.42	1.17
Nitrate	0.25	2.43	2.30	2.64

[a]Intra-day: running three times within 24 hours.
[b]Inter-day: running six times within successive 7 days with at least 24-hour intervals.
[c]Reproducibility was evaluated by the relative standard deviation (RSD, %).

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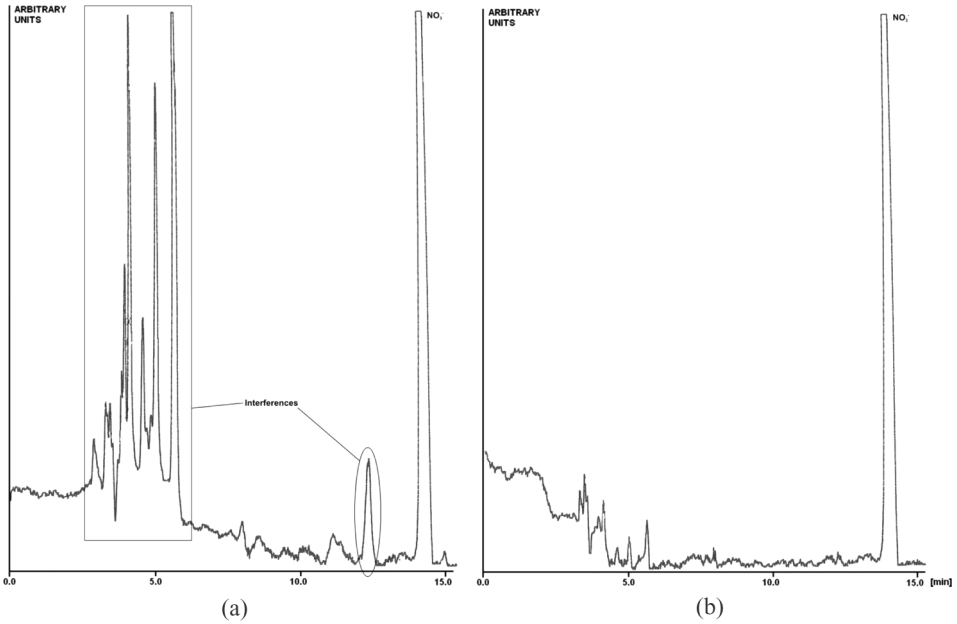

FIGURE 1 (a) Chromatogram of spinach samples extracted without addition of activated charcoal. **1** (b) Chromatogram of spinach samples extracted with addition of activated charcoal.

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charcoal as can be observed in Fig. 1b. Both extraction techniques gave almost similar areas for the nitrate peak. Standard addition of nitrite indicated that this compound was not detected in the analyzed vegetables. Extraction with activated charcoal gave cleaner chromatograms, without loss of nitrate and nitrite content, thus it was chosen because it contributes to preserve the chromatographic column.

Further recovery studies were performed adding to the sample an equal amount of activated charcoal. The recovery percentage of nitrate and nitrite spiked into vegetable samples were in the range of $97.8 \sim 108.3\%$ and $97.5 \sim 101.4\%$, respectively, for nitrate and nitrite (Table 2). The

TABLE 2 Recoveries of Nitrate and Nitrite Spiked into Fresh Spinach and Lettuce Samples

	Recovery (%) and Standard Deviation[c]	
Spike Level (mg L^{-1})	Nitrate	Nitrite
2.24[a]	108.3 ± 4.9	101.6 ± 1.0
4.25[a]	100.6 ± 3.8	97.8 ± 3.5
4.34[b]	97.5 ± 0.46	101.4 ± 1.0

[a]The content of nitrate in unspiked lettuce was 2.91 mg L^{-1}; nitrite was not detected.
[b]The content of nitrate in unspiked spinach was 3.79 mg L^{-1}; nitrite was not detected.
[c]Average of triplicate assays.

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TABLE 3 Comparison of Weight Loss and Moisture Content for Fresh, Freeze-dried, Oven Dried and Frozen Spinach and Lettuce Samples

Pretreatment	Fresh		Freeze-dried		Oven Dried		Frozen	
	Frozen 6 h and analyzed in less then 24 h after collection		Analyzed after 36 h of lyophilisation and sifted through a pore less then 500 μm		Analyzed after 48 h of force air oven drying at 70°C and sifted through a pore less then 500 μm		Analyzed after 2 weeks of freezing	
	S	L	SFd	LFd	SO	LO	SF	LF
Initial weight (g)	100	100	100	100	100	100	100	100
Final weight (g)	100	100	10.05	4.07	9.86	4.55	100	100
Moisture (%)	88.9	94.7	11.7	14.6	12.9	15	90.4	94.4

S – fresh spinach; L – fresh lettuce; SFd – freeze-dried spinach; LFd – freeze-dried lettuce; SO – oven dried spinach; LO – oven dried lettuce; SF – Frozen spinach; LF – frozen lettuce.

average recovery for nitrate was 102.1% and for nitrite was 100.3%, indicating the method is quite accurate. These recovery percentages were similar to those from literature.[26] This HPLC method was chosen to evaluate the effect of sample pretreatment because it is fast, sensitive, and accurate.

Effect of Sample Pretreatment

Weight loss and moisture content for spinach and lettuce samples (fresh, freeze dried, oven dried, and frozen) were evaluated (Table 3). Fresh lettuce presents around 95% moisture whereas fresh spinach presents less moisture around 89%. No significant differences were observed in the moisture content of fresh and frozen samples. Freeze dried and oven dried spinach samples presented around 12% moisture and for lettuce samples the levels were around 15%. Moisture content of the samples was important for quantification of nitrate content.

The effects of freeze dried, oven dried, and frozen pretreatments on nitrate content of spinach and lettuce samples are shown in Tables 4 and 5, respectively. The results of this study indicate that there is a wide range of concentrations in the experimental determination of nitrate based on the pretreatment of the sample used. ANOVA analysis at the 95% confidence level shows that there is significant difference between the four sample pretreatments for spinach (Table 4) and for lettuce (Table 5). The results from fresh and two weeks frozen samples were closely comparable to each other as indicated by the Ducan test. Prasad & Chetty observed minor loss of nitrate content on seven days of frozen samples that was attributed to any microbial action that took place during the period when the samples are removed from freezing and are thawed.[6] However, in the present work, samples were not thawed because during this process they exude water and consequently lose nitrates (results not shown).

Freeze dried and oven dried spinach samples presented significantly lower nitrate content (Duncan test $p < 0.05$) when compared with fresh and frozen spinach samples. Concerning lettuce, freeze dried and oven

TABLE 4 Statistics for Variation of Spinach Pretreatments

Pretreatment	Fresh	Freeze Dried	Oven Dried	Frozen	F test
Mean (mg kg^{-1})	1284^a	1118^b	806^c	1272^a	49.3
Std dv.	19.9	37.1	71.7	18.1	
Std err.	9.93	18.6	35.8	10.4	

Letters $^{a-c}$ indicate significant differences at $p < 0.05$ in the Duncan test.

Nitrate quantification by HPLC/UV, extraction variables held constant (extraction temperature 80°C and extraction time 20 min) except sample size that was 0.25 for fresh and frozen samples and 0.025 for freeze-dried and oven dried samples, to fall within the linear range of calibration curve.

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TABLE 5 Statistics for Variation of Lettuce Pretreatments

Pretreatment	Fresh	Freeze Dried	Oven Dried	Frozen	F test
Mean (mg kg^{-1})	972.5^a	1706^b	1337^c	1043^a	69.1
Std dv.	36.5	49.9	58.4	14.4	
Std err.	21.1	22.3	26.1	22.7	

Letters $^{a-c}$ indicate significant differences at $p < 0.05$ in the Duncan test.

Nitrate quantification by HPLC/UV, extraction variables held constant (extraction temperature 80°C and extraction time 20 min) except sample size that was 0.25 for fresh and frozen samples and 0.025 for freeze-dried and oven dried samples, to fall within the linear range of calibration curve.

dried samples presented significantly higher nitrate content. Probably because freeze dried and oven dried lettuce was a light powder difficult to weigh. On the other hand, oven dried spinach containers presented residue of evaporated water that exudates from the vegetable that was difficult to remove and probably retained nitrate from the sample, resulting in lower nitrate content.

No nitrite was detected in either fresh spinach or lettuce. Additionally, nitrite was not detected in freeze dried, oven dried, and frozen samples. This is not surprising because it has been shown that nitrite concentrations in fresh, well stored vegetable tissues are extremely low and, under the frozen storage of vegetables, nitrite accumulation was inhibited.[4] Our results indicate that freeze dry and oven dry storage also inhibited nitrite accumulation.

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CONCLUSIONS

Activated charcoal was efficient to remove chromatographic interferences from vegetable matrices. The range of results for each experimental set of fresh, freeze dried, oven dried, and frozen spinach and lettuce samples indicates the need to standardize appropriate pretreatment for nitrate determination. Similar nitrate concentrations were obtained for fresh and two weeks frozen samples. Freeze drying and oven drying pretreatment of the spinach and lettuce material was inappropriate. No nitrite was detected in either fresh, freeze dried, oven dried, and frozen spinach or lettuce.

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