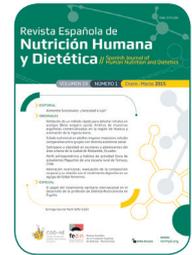


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ORIGINAL

Validation of a rapid method for detecting nitrate in chard (*Beta vulgaris cycla*). Analysis of Spanish commercial samples marketed in the Region of Huesca, Spain, and estimation of the daily intake

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KEYWORDS

Nitrate;
Vegetables;
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➤ Validation of a rapid method for detecting nitrate in chard (*Beta vulgaris cycla*). Analysis of Spanish commercial samples marketed in the Region of Huesca, Spain, and estimation of the daily intake

ABSTRACT

Introduction: This paper presents the validation of a rapid method for the determination of nitrate content in chard samples, in order to determine, during two periods (winter and summer of 2012), the current levels of nitrate in this typical vegetable, and the toxicological risk associated with this intake.

Material and Methods: A rapid colorimetric determination of nitrate in chard samples by nitration of salicylic acid was validated. The validated method was applied to analyze the content of nitrate in 56 chard samples marketed in Huesca (Spain) and collected in winter and summer seasons, and the toxicological risk associated with the intake for adult and children population was evaluated.

Results: The method was specific and robust enough for the required purposes. The main performance characteristics of the method were: limits of detection and quantitation of 0.29 mg L⁻¹ and 0.59 mg L⁻¹, respectively; recoveries from 80.0% to 107.4%; and coefficients of variation lower than 11.4%. The detected mean nitrate content was 2293 mg kg⁻¹ and there was evidence of risk only for extreme consumers (adults and children), especially in winter period.

Conclusions: A high percentage of chard samples with a considerable concentration of nitrate were found. Taking into account the estimated dairy intake of nitrate associated with them, it could be recommended to establish a regulatory limit of nitrate to chard, a vegetable of important consumption in Spain.

➤ **Validación de un método rápido para detectar nitratos en acelgas (*Beta vulgaris cycla*). Análisis de muestras españolas comercializadas en la región de Huesca y estimación de la ingesta diaria**

PALABRAS CLAVE

Nitratos;
Vegetales;
Contaminación;
Ingesta diaria;
Riesgo toxicológico.

RESUMEN

Introducción: En este trabajo se presenta la validación de un método rápido para la determinación del contenido de nitratos en muestras de acelga, con el fin de determinar durante dos periodos (invierno y verano de 2012) los niveles de nitratos en esta hortaliza típica y el riesgo toxicológico asociado con este consumo.

Material y Métodos: Se validó un método para la determinación colorimétrica de nitratos en muestras de acelga basado en la nitración del ácido salicílico. Una vez validado el método, se aplicó para analizar 56 muestras de acelgas del mercado de Huesca (España) recogidas en las temporadas de invierno y verano, y se valoró el riesgo toxicológico asociado al consumo para la población española adulta e infantil.

Resultados: El método fue específico y robusto para los fines requeridos. Las principales características del método fueron: límites de detección y cuantificación de 0,29 mg L⁻¹ y 0,59 mg L⁻¹, respectivamente; recuperaciones de 80,0% a 107,4%; y coeficientes de variación inferiores a 11,4%. El contenido medio de nitratos detectado fue de 2293 mg kg⁻¹ y sólo hubo evidencia de riesgo para los consumidores extremos (adultos y niños), especialmente en el invierno.

Conclusiones: Se encontró un alto porcentaje de muestras de acelgas con una concentración considerable de nitratos. Teniendo en cuenta la ingesta diaria estimada de nitratos asociada a ellas, se recomendaría establecer un límite reglamentario de nitratos en acelgas ya que es una hortaliza de consumo importante en España.

CITA

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INTRODUCTION

Vegetables constitute the major source of dietary nitrates. The accumulation in plants varies widely between species, and there are several plants that possess higher tendency to accumulate nitrate¹. Typically, leafy vegetables contain higher nitrate concentrations compared to roots and fruits. The nitrate content in vegetables may be influenced by factors related to the plant (variety, species and maturity), and to the environment (temperature, light intensity, fertilizers, water used, and deficiency of some nutrients)². In this sense, there are many bibliographic references that report higher nitrate content in leafy winter vegetables compared to the summer crops.

The significance of nitrate to human health derives from the fact that nitrate can be converted *in vivo* to nitrite which shows toxic effects³. In this regard, maximum limit concentrations of nitrate for certain vegetables (lettuce and

spinach) are established by the European Union⁴. Nitrate levels in lettuce and spinach have been controlled a long time ago in countries of the European Union and around the World^{3,5-7}, but there are few data about chard, one of the most widely consumed vegetables in Spain.

Many analytical techniques as spectrophotometry, potentiometry, ion chromatography, polarography, capillary electrophoresis and high-performance liquid chromatography have been used for quantification of nitrates in vegetables samples⁸, but many of them have been often lengthy and expensive.

Taking into account the above, the aim of this study was the validation of a rapid method for the determination of nitrate in chard samples, a typical vegetable of important consumption in Spain, in order to know, in a fast way, the current levels of nitrate in chard samples marketed in Huesca (Spain) and to determine the toxicological risk associated with this intake.

MATERIAL AND METHODS

Reagents and Instrumentation

Nitrate calibration standard solutions were prepared from a stock solution of 1000 mg L⁻¹ of nitrate by Panreac (Barcelona, Spain) in distilled water and stored in capped amber vials at 4°C. Salicylic acid 99% PS, sodium hydroxide PA-ACS-ISO and sulphuric acid 93-98% were supplied by Panreac (Barcelona, Spain). A separate blank was prepared by mixing 0.8 mL of sulphuric acid concentrated (93-98%), 19 mL of sodium hydroxide 2N and 0.25 mL of distilled water. The absorbance of samples was measured using a double beam UV/Visible scanning Spectrophotometer (Unicam UV/Vis Spectrometer UV2).

Samples Preparation

The samples were acquired in local markets and large supermarkets of the Huesca region. A sample consisted of 1 kg of vegetable. Samplings were instructed to keep the samples and to take them to the laboratory to be prepared.

Prior to analysis, non-edible parts of the sample were removed and the whole samples were chopped and frozen, before the analysis, they were homogenized in a blender while frozen. The nitrates content of chard was determined in two different periods (winter and summer). The first sampling was performed from October 1 to March 31, and the second from April 1 to September 30.

Method validation

Method optimization: The analytical method for the determination of nitrate in plant tissue was described by Cataldo *et al.*⁹ who proposed a colorimetric determination of nitrate in plant tissue by nitration of salicylic acid, after extraction with distilled water, phosphate buffer and centrifugation. Other authors proposed extraction of nitrate, only with hot water^{6,10} or methanol/water (30:70)¹¹. Based on these works, and in order to get rapid analyses of chard, a simple extraction in distilled water was studied, without centrifugation proposed. Different temperatures and presence of agitation were considered.

Absorbance of the complex formed by nitration of salicylic acid is directly proportional to the content of nitrate. Palomino *et al.*¹² showed the absorption spectrum of the chromophore, in line with this work, two different wavelengths (410 nm and 430 nm) were tested.

Specificity: On one hand, aqueous calibration curves (n=3) were constructed from several standard solutions at six

concentrations of nitrate from 0.5 to 5.0 mg L⁻¹; on the other hand, standard addition curves (n=3) were prepared from chard samples spiked from 1.5 to 5.0 mg L⁻¹. To verify the absence of interfering substances, the slopes of the aqueous calibration and the standard addition curves were compared by applying an ANOVA test. A *p*-value < 0.05 was considered statistically significant. The aqueous calibration curves were employed to determine the nitrates level of chard commercial samples, when there were no significant differences.

Calibration curves: Standard solutions at six concentrations including zero, spaced across the working range (0.5 to 2.5 mg L⁻¹) were processed for six times, and calibration curves were constructed in order to know the linearity of the method. Slope and coefficient values (R) were measured.

Detection and quantification limits: The detection and quantification limits were determined by analyzing ten blanks, corresponding to mean value plus three times and ten times the standard deviation, respectively.

Repeatability and Reproducibility: Repeatability and reproducibility of the analytical method were assessed. The analyses of each level of the aqueous calibration curves were performed in triplicate on three successive days, and the coefficient of variation for intra-day and for inter-day assays were valued. In the same way, repeatability and reproducibility of spiked samples (1.5 to 5.0 mg L⁻¹) were analysed.

Accuracy: For the period in which samples were examined, nitrate reference test materials were not available (spinach, lettuce), so it was decided to fortify real samples. Chard leaves samples were spiked with four different points of fortification, including zero (1.5 to 5.0 mg L⁻¹), and subjected to extraction process proposed in this paper. Accuracy was evaluated by the comparison of the theoretical values of the nitrate spiked samples and the values obtained in triplicate. The analytical method was considered accurate if the recovery percentages obtained were within 80–110%¹³.

Stability: The solution of salicylic acid may be unstable⁹. In order to check this fact, some extracts of chard samples (n=3) were analysed using the solution prepared just at the moment of analysis and results were compared to those carried out with solutions prepared one week before (n=3). The acceptance criterion was a response between 95% and 105% of the initial one¹⁴.

Dietary exposure estimates

The daily intake of nitrate through chard was calculated from the median concentration of nitrate in samples and

the weight consumed. In this work, the daily consumption data from Spanish population analyzed (children 7-12 years, adults and extreme consumers) came from the Spanish Food Safety Authority¹⁵.

RESULTS

Validation of Analytical Method

Experimental and instrumental conditions were optimized to get a rapid method to determine nitrate in chard. Two different wavelengths (410 nm and 430 nm) were tested in order to determine the best absorption conditions (Figure 1). As can be seen, the complex formed absorbed maximally at 410 nm. The calibration curve obtained at 410 nm presented better sensitive (slope 0.0785 versus 0.0614) and less uncertainty in their values so, it was decided to work at this wavelength.

All the changes made to the initial proposed method were shown in Table 1. As can be observed, experiments showed better nitrate extraction if samples (fresh or defrosted) were mixed with distilled water, stirred and heated (70°C).

The final analysis protocol was as follows. The sample (1.5 g) was weighed and transferred into a 100 mL beaker and 50 mL of distilled water were added. The beaker was placed on a stirred hotplate for 30 minutes without boiling (70°C) and finally adjusted up to 50 mL with distilled water in a volumetric flask. After filtration, an aliquot of 0.2 mL was transferred to a test tube and mixed thoroughly with 0.8 mL of salicylic acid 5% (w/v) in sulphuric acid (93-98%). After 20 minutes at room temperature, 19 mL of sodium hydroxide 2N were added to raise the pH above 12. The samples were cooled at room temperature and absorbance was measured at 410 nm.

Specificity study was shown in Figure 2. The slopes of both, aqueous calibration curves (standard solutions of nitrate from 0.5 to 5.0 mg L⁻¹), and standard addition curves

Figura 1. Calibration curves at 410 nm and 430 nm.

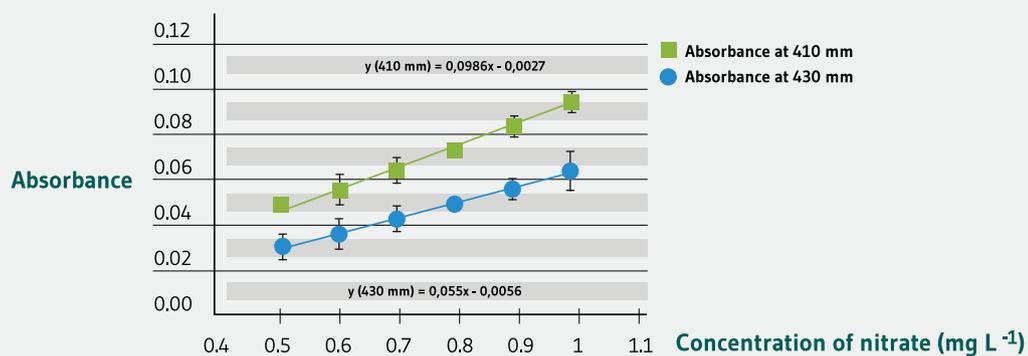


Tabla 1. List of experiments that were carried out to get faster analysis.

	Sample	Temperature	Stirring	Weight(g)	Nitrate (mg kg ⁻¹)
Exp1	fresh	70 °C	Yes	1.5	2704.9
Exp2	fresh	70 °C	No	1.5	2468.5
Exp3	defrosted	25 °C	No	1.5	1696.8
Exp4	defrosted	25 °C	Yes	1.5	2236.9
Exp5	defrosted	70 °C	Yes	1.5	2702.8

(extracts of chard samples spiked from 1.5 to 5.0 mg L⁻¹), were compared by applying an ANOVA test, there were no statistically significant differences between slopes ($p > 0.05$) so, no matrix effect was observed.

The detection and quantification limits (mean plus three times and ten times the standard deviation), were 0.29 mg L⁻¹ (967 mg kg⁻¹) and 0.59 mg L⁻¹ (1833 mg kg⁻¹), respectively.

Repeatability and reproducibility of the analytical method were assessed. The analyses of each level of the aqueous calibration curves in triplicate on three successive days showed coefficient of variation values from 0.5% to 5.4% for intra-day and from 0.7% to 11.4% for inter-day assays. In the

same way, spiked samples (1.5 to 5.0 mg L⁻¹) were analysed, and these values were from 0.2% to 4.6% and from 1.7% to 8.7%, respectively (Table 2). All coefficient of variation were lower than 15%¹³.

The accuracy study showed recovery percentages from 80.0% to 107.4%, and a degradation phenomenon was observed during storage of one week at 20°C in darkness, with a response of 86.1% of the initial one.

Analysis of commercial samples

Table 3 shows the nitrate contents in 56 samples collected in winter and summer seasons and marketed in Huesca,

Figura 2. Specificity study with aqueous calibration and standard addition.

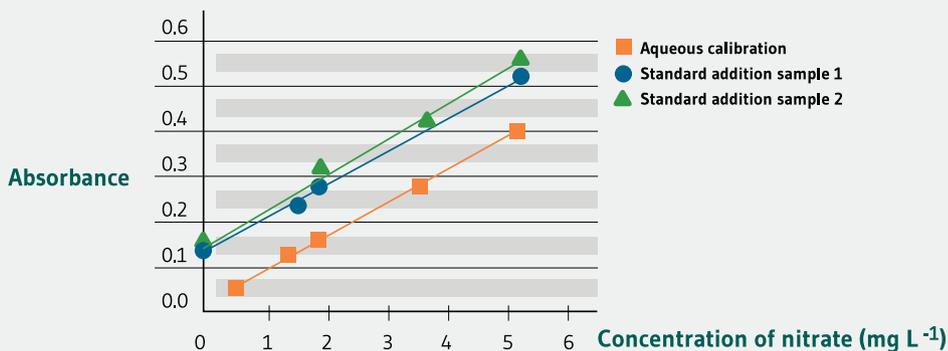


Tabla 2. Coefficient of variation (CV) intra-days and inter-days for analytical method.

NO ₃ (mg L ⁻¹)	CV (%) Intra-day			CV (%) Inter-days
	1	2	3	
0.5	1.1	5.4	4.8	11.4
1.5	3.3	1.2	1.2	7.2
1.9	0.6	0.6	1.3	2.8
3.5	0.8	1.4	1.1	0.7
5.0	0.5	0.8	0.7	1.2
NO ₃ added (mg L ⁻¹)	CV (%) Intra-day			CV (%) Inter-days
1	2	3		
0.0	4.6	2.0	2.1	4.7
1.5	3.5	3.3	2.5	7.1
1.9	1.3	0.6	1.2	8.7
3.5	2.0	0.8	3.1	3.0
5.0	0.4	0.5	0.2	1.7

Spain. To calculate the content of nitrate in chard samples, they were taken into account the dilution carried out on the extract (1:100), the volume of extract prepared (50 mL) and the weight of sample used (1.5 g). Samples with an absorbance signal less than detection limit were classified as samples with nitrate content equal to 967 mg kg⁻¹ (*worst case*) and samples with an absorbance signal less than quantification limit, equal to 1833 mg kg⁻¹ (*worst case*).

The 56 samples analysed presented a mean and a median nitrate content of 2293 mg kg⁻¹ and 1833 mg kg⁻¹, respectively. These values were 2399 mg kg⁻¹ and 1930 mg kg⁻¹, for samples cultivated in winter, and 2186 mg kg⁻¹ and 967 mg kg⁻¹ for summer crops.

Taking into account the existing regulatory limits, these values were, in all cases, below to the maximum limits established by the European Commission for similar vegetables (*Spinacia oleracea*), 3000 mg kg⁻¹ and 2500 mg kg⁻¹ for winter and

summer, respectively. However, 15 samples (26.8%) exceeded these values, seven winter samples (25.0%) and eight summer samples (28.6%).

Dietary exposure estimates

The estimated daily intake (EDI) of nitrate through chard is shown in Table 4.

Considering an acceptable daily intake (ADI) for nitrate of 3.7 mg kg⁻¹ (body weight) day for a 60 kg adult¹⁶, the nitrate daily intake through chard collected in winter was unacceptable only for extreme consumers, with a relationship EDI/ADI of 107.3% for children and 90.3% for adults. When samples were collected in summer, risk decreased (53.8% and 45.1% for children and adults). No risk was observed for children and adult mean consumers (1.9% and 4.1% for summer and winter period, respectively).

Tabla 3. Nitrate contents in chard samples collected in winter and summer seasons, marketed in Huesca, Spain.

Season	N° samples	Nitrate content (mg kg ⁻¹)			CV (%)
		Range	Mean	Median	
Winter	28	967–5999	2399	1930	4.9–24.5
Summer	28	967–9093	2186	967	0.7–17.8

Tabla 4. Estimation of the nitrate dietary intake through chard.

Season	Consumption (g day person ⁻¹)				EDI (mg kg day ⁻¹)				EDI/ADI (%)			
	Adults		Children		Adults		Children		Adults		Children	
	Mean	High level	Mean	High level	Mean	High level	Mean	High level	Mean	High level	Mean	High level
Winter												
Summer	5.2	118.5	2.6	71.0	0.2	3.3	0.2	4.0	4.1	90.3	4.1	107.3
					0.1	1.7	0.1	2.0	1.9	45.1	1.9	53.8

Adult (17 years and over) weight: 68.5 kg

Children (7-12 years) weight: 34.5 kg

High level: percentile 97.5%

DISCUSSION

These results revealed the existence in the Spanish market of a high percentage of chard samples with a considerable concentration of nitrate (Mean and median nitrate content of 2293 mg kg⁻¹ and 1833 mg kg⁻¹, respectively). According to these results, other studies performed in Spain, noted the existence of chard contaminated with similar levels of nitrate. Pardo-Marín *et al.*¹⁷ determined the presence of nitrate in chard by means of liquid chromatography obtained a median nitrate content of 1597 mg kg⁻¹. According to other revisions, chard presented similar or even higher nitrate content compared to other vegetables. Mor *et al.*³ showed a mean nitrate content of 1132 mg kg⁻¹ for spinach and 1439 mg kg⁻¹ for lettuce marketed in Turkey. Meanwhile, Tamme *et al.*⁷ showed mean nitrate levels of 2337 mg kg⁻¹ and 3023 mg kg⁻¹ for spinach and lettuce in Estonia. The median nitrate content in green salads, spinach and lettuce included in the Italian diet were 1720 mg kg⁻¹, 1954 mg kg⁻¹ and 1440 mg kg⁻¹ respectively⁵, and 2684 mg kg⁻¹ and 1747 mg kg⁻¹ in lettuces and spinach from Sweden⁶.

As can be seen in this study, chard samples collected in winter accumulated more amount of nitrate than those collected in summer. The influence of seasonal variation of nitrate contents in leafy vegetables has been documented in literature. The lettuce and spinach from Estonian market had a 22% and 24% over nitrate levels when grown in winter than in summer⁷. Lettuces grown in UK, showed a mean nitrate level of 3124 mg kg⁻¹ and 2382 mg kg⁻¹ during winter and summer months respectively¹⁸. In Spain, spinach analyzed during winter, showed median concentration levels of nitrate in the order of 1755 mg kg⁻¹ versus 1269 mg kg⁻¹ for spinach collected in summer¹⁷.

Regarding the dietary exposure estimates, these results were similar to other Spanish data. The mean consumption of chard of Valencia market supposed a nitrate daily intake of 3.5% of the ADI, in case of adult consumers, and 1.7% for children. The EDI for extreme consumers was 78.9% of the ADI¹⁷.

CONCLUSIONS

The method for analysis of nitrate in chard explained in this paper was specific, stable, robust, precise and accurate, and the sample preparation procedure took less than one hour, so this analytical method could be a low cost and rapid procedure to determine nitrate in chard.

In the Spanish market, there were chard samples with similar or even higher nitrate levels compared to other leafy vegetables (according to other revisions), and there were evidence of risk for extreme consumers in winter period.

Although this study is focused on the area of Huesca, and the samples analyzed were not nationally representative, the authors conclude that there may be a toxicological risk associated with the consumption of chard, and it could be recommended to establish a regulatory limit of nitrate to chard, as well as for other vegetables of important consumption in Europe.

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COMPETING INTERESTS

None of the authors had any conflict of interest from a financial, personal, or professional aspect in relation to the findings of this study.

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