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SELECTION OF ANALYTICAL METHODS FOR THE DETERMINATION OF NITRATE AND NITRITE IN VEGETABLES ⁽¹⁾

Among the analytical methods for determination of nitrate and nitrite in vegetables three were chosen and studied in terms of accuracy, precision, sensitivity, detection limit, ease of performance, safety and practical convenience for routine use. Some suitable modifications have been introduced and the two methods selected for nitrate and nitrite determination are described in detail.

1 — INTRODUCTION

It is well known that some plants like spinach, lettuce or beet are generally rich in nitrate; this level becomes higher if nitrate fertilizers are used. As nitrate may be reduced to nitrite in fresh products after harvest, the nitrite being toxic in low levels, the interest of an accurate determination of these species has increased in the last decade.

The primary toxicity of nitrite is manifested through oxidation of iron in hemoglobin from the ferrous to the ferric form, and there have been reports of infants with ages between two and twelve months experimenting more or less severe symptoms of methaemoglobinaemia after eating spinach [1, 2]. Furthermore, in acid medium, nitrite can react with amines of nucleic acids to form nitrosamines which can originate alterations in the genetic code. This type of reaction is general: N-nitroso compounds are formed by reaction of nitrite with secondary amines [1,3,13]. Taking into account the great variety of amines of this type existing in food and as the rate of formation of N-nitroso compounds is proportional to the square of nitrite concentration, efforts should be made to reduce the amount of nitrite ingested. This is a difficult task because it is usual to fertilize in excess to increase the yield and quality of crops. Moreover, in spite of its disadvantages and since there is no substitute, the nitrite ion is used as a preservative and bactericide in smoked meat to prevent the proliferation of *Clostridium botulinum* and improve its colour and taste.

The World Health Organization has established provisional limits to the levels of nitrate and nitrite in some kinds of food — 500 mg/kg for nitrate (as NaNO₃) and 200 mg/kg for nitrite (as NaNO₂) for canned and smoked meat [42]. This Organization has also considered that nitrate and nitrite ingested by man should not exceed a maximum daily limit of 5 mg/kg and 0.2 mg/kg body weight [42].

Owing to the necessity of respecting such limits and since there are no official methods for nitrate and nitrite determination in plants, the aim of this work is to select, among the methods reported in the literature, those which can be easily used in control and research laboratories without auto-analysers.

(17 Part of this work has been presented at the «1.º Encontro Nacional de Química» — Lisboa — Portugal (1978).

Considering the importance of spinach in children's diet this was the vegetable chosen for our study although the conclusions can be easily extended to any other type of plants.

Different methods for nitrate determination can be found in the literature; such methods, mainly applied to vegetables, include polarography [4-6], potentiometry with nitrate selective electrode using different modes of extraction [7-11], spectrophotometry in the ultraviolet range [12, 28] applicable to the determination of nitrate and nitrite and spectrophotometry in visible range using the strong colour of the azo compound formed by reaction of the nitrite with aromatic amines [14, 20-24, 29]. Nitrate ion is indirectly determined using the same methods after having been quantitatively reduced to nitrite using a reducing cadmium column [36, 37]. Considering the carcinogenic character of the aromatic amines some authors [38] use amines with sulfonic groups to reduce this inconvenience [39-41], an important improvement for routine methods.

The spectrophotometric methods applicable only to the nitrate ion should also be mentioned; their application is based on the reaction of this ion with diphenylamine [25] and diphenylamine and *p*-diaminodiphenylsulphone [35], with Re(V) and α -furildioxime [27, 33], with salicylic acid in alkaline medium [15, 16], with phenazone [17, 29], with 2,4-xyleneol [18], with 2,6-xyleneol [19, 26], with brucine [32] or phenoldisulfonic acid [34].

Another method to be considered is the steam distillation method [30] for determination of ammonium, nitrate and nitrite.

Attending to their accuracy, precision, sensitivity, detection limit, ease of performance, safety and practical convenience for routine use, the following three methods were selected and tried in laboratory after suitable modifications had been introduced.

- 1 — Nitrate selective electrode potentiometry
- 2 — Cleve's Acid reagent spectrophotometry
- 3 — Steam distillation

After experimental study of these three methods, the first has been selected for determination of nitrate and the second for determination of nitrite.

Each of these methods is described in detail including the modifications introduced by us on account of the great interest of this subject and in order to make known the techniques extending them to the analytical chemistry control laboratories.

2 — DETERMINATION OF NITRATE IN SPINACH

(Selective electrode potentiometry)

We have adopted the method described by MILHAM *et al.* [10]; its main advantages compared with the non automatic colorimetric methods are the shorter time of analysis and an easier preparation of standards and samples.

The extracting solution for samples has also complexation and precipitating effects, thus making it possible to eliminate most interferences [10].

2.1 — EXPERIMENTAL

2.1.1. — EQUIPMENT AND REAGENTS

- Orion Nitrate «Specific» electrode, Model 92-07 (Orion Research Incorporated, Cambridge Massachusetts, U.S.A.).
- Reference Electrode (SCE), Corning n.º Cat. 476109.
- Coleman pH meter (model 38A) with an accuracy of ± 0.1 mV.
- Salt bridge saturated in potassium sulphate and 6% in agar-agar.
- Kenwood mixer.
- Standard solution 1000 ppm in nitrate — prepared with potassium nitrate *p.a.*
- Extracting solution — 0.010 M in aluminium sulphate, 0.010 M in silver sulphate, 0.020 M in boric acid and 0.020 M in sulphamic acid, adjusted to pH = 3.0 with 0.1 M potassium hydroxide.

2.1.2 — PROCEDURE

CALIBRATION CURVE

In order to establish the calibration curve, standards were prepared containing between 10 and 1000 ppm of NO_3^- diluted 1:1 with extracting solution. It was found that the slope of the straight line $E = f(\log c)$ was reproducible with time, although its value was higher than that predicted by Nerst equation.

All the readings were done by shaking the solution with a magnetic stirrer during 3 minutes and reading the value 2 minutes after stirring had stopped.

PREPARATION OF THE SAMPLES

Fresh leaves of spinach were washed, air dried and ground. Samples of 5 to 10 g were weighed and nitrate was then extracted using 100 ml of extracting solution and 50 ml of water. After 1 hour stirring, the volume of the solution was made up to 200 ml. The sample was filtered and the nitrate level determined by direct potentiometry.

Notes

It should be pointed out that relatively to MILHAM *et al.*'s paper [10], the following changes have been introduced:

1 — The reference electrode Radiometer 601 with saturated solution of sodium sulphate used by the authors has not been used. A saturated calomel electrode and a salt bridge of saturated potassium sulphate and 6% in agar-agar were preferred instead; this proves to be more economical. Saturated sodium sulphate solutions made 6% in agar-agar do not solidify at room temperature.

2 — The recommended four minute extraction of 100 mg of ground plant with 5 ml of extracting solution and 5 ml of water has not been followed as 100 mg was considered to be an insufficient sample; 1 hour extraction was adopted although a 30 minute period would be enough. In the first case a more easily filtered solution is obtained and the time of the experiment is not remarkably increased since it is possible to extract the samples while the standards are being prepared

3 — DETERMINATION OF NITRITE IN SPINACH

(Cleve's acid reagent spectrophotometry)

The spectrophotometric method used was the one described by ADRIAANSE and ROBBERS [38] which involves the use of a non-carcinogenic product.

3.1 — EXPERIMENTAL

3.1.1. — EQUIPMENT AND REAGENTS

— Visible and ultraviolet spectrophotometer, Hitachi-Perkin-Elmer, model 139, using 1 cm cells.

— Kenwood mixer.

— Electromagnetic stirrer.

— Buffer solution pH = 9.6 — 9.7 (0.67 M in NH_4Cl and NH_3).

— Diluted buffer solution 1:10 in volume).

— Sodium hydroxide solution 2.5 M.

— 1,7 Cleve's acid solution — 60 mg of Cleve's acid (BDH) were dissolved in 50 ml of hot distilled water. After cooling, 3-4 g of powdered zinc were added to the light red solution and this was stirred for 2-3 minutes. After filtering into a brown bottle, 50 ml of acetic (99-100%) were added. The solution was shaken and stored in a cool place.

— Sulphanilic acid solution — 600 mg of sulphanilic acid were dissolved in 50 ml of hot distilled water. 50 ml of acetic acid (99-100%) were added and the solution was shaken and stored in a brown bottle.

— Fundamental solution for MILHAM'S method [10]. This solution was prepared mixing equal parts of the above referred Cleve's acid solution and sulphanilic acid solution.

— Extracting solution:

50 g of CdCl_2 , 2 1/2 H_2O and 50 g of BaCl_2 , 2 1/2 H_2O were dissolved in about 1 liter of distilled water. pH was adjusted to 1 with HCl and diluted to 1 liter with distilled water.

— Nitrite stock solution — 0.1500 g of sodium nitrite were dissolved in distilled water in a volumetric flask (1000 ml). Add 5 ml of buffer solution and make up to volume.

— Activated charcoal — Darco G 60 — Fluka. Other types of charcoal did not give reproducible results.

3.1.2. — PROCEDURE

CALIBRATION CURVE

20 ml of nitrite stock solution were transferred into a 500 ml volumetric flask, 5 ml of buffer solution were added and the solution was made up to volume. 0.0, 5.0, 10.0, 15.0, 25.0, 50.0 and 75.0 ml aliquots were transferred into 200 ml volumetric flasks, 10 ml of buffer solution were added and the solution was made up to volume. 10 ml of this solution were transferred to a 25 ml volumetric flask, 10 ml of the «fundamental solution» were added and diluted to volume with distilled water.

The flasks were placed in a water bath at 25-30° for 30 minutes and the absorbance at 530 nm was measured with a spectrophotometer using 1 cm cells.

SAMPLE PREPARATION

Following the method proposed by SCHALL and HATCHER [20], 20 g of spinach previously washed, air dried and ground were transferred to a 200 ml volumetric flask; 100 ml of extracting solution, 1 g of Fluka activated charcoal (Darco G-60) and 50 ml of distilled water were added. The flask was shaken mechanically for 1 hour and then 16 ml of sodium hydroxide (2.5 N) were added and diluted to volume with distilled water. The solution was immediately filtered, first through a fast filter paper and then through a slow filter paper. 5 ml of buffer solution

(NH₃/NH₄Cl) were transferred to a 50 ml volumetric flask, diluted to volume with clear filtrate and mixed. 15 ml of this solution were transferred to a 25 ml volumetric flask, 10 ml of the method's «fundamental solution» were added and the nitrite content determined. Although the extracting solution used is acid the nitrite ion is not lost according to the reaction $3\text{HNO}_2 \rightleftharpoons \text{H}^+ + \text{NO}_3^- + 2\text{NO} + \text{H}_2\text{O}$ because the nitrate level is much higher than the nitrite one.

Notes

1 — SCHALL and HATCHER [20] extraction method was preferred to that indicated by ADRIAANSE and ROBBERS [38], because it is not necessary to add the clarifying solution of zinc sulphate and potassium ferrocyanide, thus simplifying operations.

Table 1
Results for standard solutions

Analytical method	Mean Deviation of standards	Detection limit ($\mu\text{g/l}$) in nitrogen	Sensitivity (slope of calibration straight line)	Correlation coefficient
Selective electrode potentiometry (NO_3^-)	2.0 mV	14.0	$-65.3 \pm 0.5 \text{ mV}^{(a)}$	0.999
Cleve's reagent spectrophotometry (NO_2^-)	0.005 absorbance units	3.0	0.9 ppm^{-1}	1.000
Cleve's reagent spectrophotometry (NO_3^-)	0.010 absorbance units ^(b)	3.0	0.9 ppm^{-1}	0.999
Steam Distillation		175		

a) The slope of the straight line $E = f(\log [\text{NO}_3^-])$ is -65.3 ± 0.5 , a value which is somewhat higher than that predicted by Nernst equation; it is, nevertheless fairly reproducible.

b) The highest value of the mean deviation for nitrate standards is due to the errors introduced by the reduction of nitrate to nitrite in the cadmium column.

2 — The addition of activated charcoal to the standards according to ADRIAANSE and ROBBERS [38] was unnecessary and therefore omitted.

This method can also be used for nitrate determination after reducing it to nitrite in a cadmium column. However the process is delayed by this operation because reproducible results can only be obtained if the flow of the solution through the column is rigorously controlled. This flow must be controlled to obtain the maximum value of absorbance, which means a quantitative reduction of nitrate ion to nitrite without reducing the latter.

The BREMER and KIENEY'S [30] steam distillation method which has also been studied experimentally and in which some alterations were introduced is not described in detail. The most important alteration consists in changing the extracting solution: instead of 1 M potassium chloride, SCHALL and HATCHER [20] extracting solution was used and so this method became more reproducible and accurate.

4 — RESULTS AND DISCUSSION

In order to determine the best conditions to apply the different methods, their accuracy, sensitivity and precision, an experimental study has been carried out on a comparative basis.

Table II

Recovery percentage of an added sample analysed by the described methods

Analytical method	Added concentration (nitrogen ppm)	Determined concentration	% Recovery
Selective electrode potentiometry (NO_3^-)	62.4	66.0	105.0 ^u
	82.1	76.0	92.6
	143.4	151.0	105.3
Cleve's Reagent spectrophotometry (NO_2^-)	0.85	0.83	97.6
Cleve's reagent spectrophotometry (NO_3^-)	0.64	0.63	98.6
Steam distillation	NH_4^+ 13.37	13.30	99.5
	$\text{NO}_3^- + \text{NO}_2^-$ 21.49	21.70	100.9
	$\text{NH}_4^+ + \text{NO}_3^-$ 28.42	29.05	102.2

4.1 — STUDIES WITH STANDARD SOLUTIONS

The selected methods have been studied in terms of reproducibility of standards, detection limits, sensitivities and correlation coefficient of the straight lines (Table I).

4.2 — STUDIES WITH SPINACH SAMPLES

4.2.1 — RECOVERY OF ADDED AMOUNTS OF STANDARD

After having studied the best experimental conditions, the recovery percentage of an amount added to the sample was determined.

4.2.2 — REPRODUCIBILITY OF THE METHODS

In order to estimate the reproducibility of each one of the methods, determinations were made for sets of ten aliquots of the same previously homogenized sample, except for the nitrite ion where four determinations were made. In this case the scattering of the results is greater because the values for fresh spinach are very close to the detection limit of the method.

Table III

Reproducibility of the analytical methods studied

Analytical method	Percentage range (in nitrogen)	σ_m	Relative error
Selective electrode potentiometry (NO_3^-)	$10^{-2} - 5 \times 10^{-2}$	0.7×10^{-3}	ca. 1%
Cleve's reagent spectrophotometry (NO_2^-)	$10^{-5} - 4 \times 10^{-5}$	0.25×10^{-5}	ca. 7%
Cleve's reagent spectrophotometry (NO_3^-)	$10^{-2} - 5 \times 10^{-4}$	5×10^{-4}	ca. 6%
Steam distillation (NO_3^-)	$10^{-2} - 5 \times 10^{-2}$	1×10^{-3}	ca. 2%

- a) It should be pointed out that in the direct potentiometry an error of ± 1 mV in the junction potential between the salt bridge and the sample and between the same salt bridge and each one of the standards leads to an error of $\pm 4\%$. So the results may be considered to be within the expected values. It should also be noted that a method which is not accurate may have good recoveries of an added amount of a standard, the inverse proposition being not necessarily true.

4.2.3 — COMPARISON OF METHODS FOR NITRATE DETERMINATION

After studying the chosen analytical methods, comparative studies were carried out between the potentiometric and the steam distillation methods on one hand, and the spectrophotometric and the steam distillation methods on the other.

NOTES:

The values given in Table IV are averages of duplicates. The dry spinach was obtained by keeping the product in an oven during 48 hours at a temperature of 70°C.

The time of 4 minutes for distillation [30] does not agree as well

with the results of the other methods as the value obtained with 8 minutes. With longer times of distillation the results are still the same.

For spinach 1) the mean value for the percentage of nitrogen as nitrate is 0.0251%, the error of the determination not exceeding 3.5% compared with average value.

For spinach 2) the mean value is 0.0321% if all the values are taken into account or 0.0304% if the value obtained by the potentiometric method using an aliquot of 20 g is neglected. The value obtained for the aliquot of 20 g using the potentiometric method may be a little too high, possibly due to the interference of chloride ion extracted together with the nitrate ion.

Table IV
Determination of nitrate in two different samples of spinach by the potentiometric method and the steam distillation method

N.° of sample ^{a)}	Extracting solution	Analytical method	Aliquot of sample	% of NO ₃ ⁻ -nitrogen in fresh spinach	% of NO ₃ ⁻ -nitrogen in dried spinach
1)	As in Ref. [10]	Selective electrode potentiometry	ca. 20 g	0.0253	0.336
	As in Ref. [10]	Steam distillation ^{b)}	ca. 20 g	0.0202 (4 min.)	0.269 (4 min.)
				0.0242 (8 min.)	0.322 (8 min.)
	BaCl ₂ + CdCl ₂ solution	Idem	ca. 20 g	0.0258 (4 and 8 min.)	0.343 (4 and 8 min.)
2)	As in Ref. [10]	Selective electrode potentiometry	ca. 20 g	0.0355	0.469
	As in Ref. [10]	Idem	ca. 10 g	0.0299	0.398
	BaCl ₂ + CdCl ₂ solution	Steam distillation ^{b)}	ca. 20 g	0.0306 (4 min.)	0.407 (4 min.)
				0.0320 (8 min.)	0.426 (8 min.)
	BaCl ₂ + CdCl ₂ solution	Idem	ca. 10 g	0.0285 (4 min.)	0.379 (4 min.)
				0.0315 (8 min.)	0.419 (8 min.)

a) Samples 1) and 2) correspond to different types of spinach. For sample 2) the leaves were lanceolate.

b) In all distillations the rate of distillation was kept constant at 7 ml/min. according to the recommended procedure [30].

Indeed the plants generally contain this ion in levels between 0.5 and 2.0% and chloride has to be lower than 10^{-1}M , otherwise it will interfere with the determination of nitrate ion using specific electrodes.

In the case studied, chloride ion did not interfere because its concentration in the solution of the extracted sample was always lower than 10^{-1}M .

It therefore appears to be more convenient to use an

aliquot of 10 g of spinach as higher aliquots increase the probability of having solutions extracted from spinach with levels of chloride exceeding the values allowed.

Comparison of the spectrophotometric method, with the above referred alterations, with the steam distillation method, has yielded the following results (Table V):

Table V
Determination of nitrate in two different spinach samples by the spectrophotometric and the steam distillation method

N.º of sample ^{a)}	Extracting solution	Analytical method	Aliquot sample	% of NO_3^- -nitrogen in fresh spinach	% of NO_3^- -nitrogen in dried spinach
1)	$\text{NH}_3/\text{NH}_4\text{Cl}$ 1:1	Cleve's reagent spectrophotometry	ca. 20 g	0.0286	0.380
	KCl 1 M	Steam Distillation ^{b)}	ca. 20 g	0.0124 (4 min.) 0.0247 (8 min.)	0.166 (4 min.) 0.328 (8 min.)
2)	$\text{NH}_3/\text{NH}_4\text{Cl}$ 1:1	Cleve's reagent spectrophotometry	ca. 20 g	0.0194	0.258
	$\text{BaCl}_2 + \text{CdCl}_2$	Idem	ca. 20 g	0.0192	0.255
	$\text{BaCl}_2 + \text{CdCl}_2$	Steam Distillation ^{b)}	ca. 20 g	0.0220 (4 and 8 min.)	0.293 (4 and 8 min.)

a) and b) see Table IV

Two extracting solutions were used here for spinach 2) that indicated by ADRIAANSE and ROBBERS [38] and SCHALL and HATCHER's solution [20] for the spectrophotometric method, the values obtained are the same (within the experimental errors). These values are identical with the ones obtained by steam distillation.

On the other hand, for spinach 1) no influence of extracting solution can be detected whether it is of ammonia/ammonium or of 1 M potassium chloride; with 8 minutes for distillation the same value is obtained both for the spectrophotometric method and the steam distillation method. The value obtained with 4 minutes for distillation is much

lower and we have noticed that this often happens when the extracting solution is KCl 1M. Using SCHALL and HATCHER's [20] method for sample preparation, the proteins are precipitated and so, in a general way, the values obtained with 4 and 8 minutes for distillation agree within the experimental errors.

Hence the three extracting solutions are all considered to be efficient and the independent analytical methods equally accurate.

4.2.4 — DETERMINATION OF NITRITE AND AMMONIUM

It was not possible to perform the comparative study of the methods to determine nitrite in spinach since

in the samples used the nitrite level was always below the detection limit of steam distillation methods. The spectrophotometric method was therefore the only one which could be used.

The determination of ammonium ion is not within the scope of this work but as it is one of the steps of the steam distillation method the values are given in table VI.

Table VI
Determination of nitrite and ammonium in two different samples of spinach

N.º of sample ^{a)}	Extracting solution	Analytical method	% of NO ₂ -nitrogen in spinach		% of NH ₃ -nitrogen in spinach	
			fresh	dry	fresh	dry
1)	NH ₃ /NH ₄ Cl 1:1	Cleve's reagent spectrophotometry	1.2x10 ⁻⁵	1.6x10 ⁻⁴	—	—
			1.2x10 ⁻⁵	1.6x10 ⁻⁴	—	—
	KCl 1 M	Steam Distillation	< 3.5x10 ⁻⁴	4.6x10 ⁻³	2.8x10 ⁻³	3.7x10 ⁻²
2)	BaCl ₂ + CdCl ₂	Cleve's reagent spectrophotometry	3.4x10 ⁻⁵	4.5x10 ⁻⁴	—	—
	BaCl ₂ + CdCl ₂	Steam	3.5x10 ⁻⁴	4.6x10 ⁻³	1.9x10 ⁻³	2.5x10 ⁻²

a) See Table. IV.

Note: The values presented for sample 1) are averages of duplicates; for sample 2) they are averages of quadruplicates. Nitrite ion levels in fresh spinach are found to be very low but should increase after the harvest.

5 — CONCLUSIONS

This study leads to the conclusion that the best method to determine nitrate is the potentiometric one. In fact, and although its detection limit is higher than of the other methods, it is still good enough for the nitrate levels usually found in spinach. On the other hand the recovery percentage of the amount added to the sample is good (Table II) and so is the reproducibility, the best of all methods (Table III). As far as accuracy is concerned all methods appear to be equally accurate, since the values obtained for a given sample are the same for the three independent methods within the experimental errors.

One great advantage of the potentiometric method relatively to any of the other two is the shorter time required for its operation, only to be compared with the spectrophotometric method with autoanalyzer, that needs a more expensive equipment.

To determine nitrite ion and noting its concentration range in spinach the spectrophotometric method seems to be accurate in terms of interferences and detection limit.

These techniques which we have selected, improved and studied in terms of accuracy, precision, sensitivity, detection limit, ease of performance, safety and practical convenience for routine use, have been used to determine nitrate and nitrite in some fresh and refrigerated vegetables.

The results obtained will be reported in a following paper.

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RESUMO

No presente trabalho faz-se uma revisão geral dos métodos descritos na literatura para a determinação dos iões nitrato e nitrito em plantas e produtos alimentares. Seleccionados três métodos que pareceram em princípio, mais convenientes, foram os mesmos estudados em pormenor em termos de reprodutibilidade, limite de detecção, exactidão, rapidez e conveniência de execução, apresentando-se alguns resultados da sua aplicação a amostras de espinafres. Com base nos resultados obtidos recomenda-se a adopção dos métodos que pareceram mais adequados para o efeito.