Determination of nitrates in biological material

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A procedure for preliminary processing of biological samples for determination of nitrates by photometric titration has been worked out.

The aqueous suspension of the homogenized sample was boiled for 2 to 3 min and subjected to electrodialysis in a special separator for 5 to 10 min. The nitrates migrated at the voltage of 12 V to the anode compartment in the electrolyte composed of 0.1 M-Na_2SO_4 and 0.01 M-H_2SO_4. Being thus separated from the interfering components (amino acids, proteins), they were determined after reduction to NH_3 by photometric microtitration.

Была разработана методика предварительной обработки биологических проб для определения нитратов посредством фотометрической титрации.

Проба после гомогенизации в виде водной суспензии 2—3 минуты варится и подвергается в течение 5—10 минут электродиализу в специальном сепараторе. В среде электролита, состоящего из 0,1 M-Na_2SO_4 и 0,01 M-H_2SO_4 напряжением 12 В ионы нитратов мигрируют к аноду, тем самым изолируясь от мешающих веществ (аминокислот, белков), а после этого их можно определить после редукции на NH_3 методом фотометрической микротитрации.

In connection with the problem of increased content of nitrates in vegetables in the last years, the necessity of effective quality control of agricultural products has increased. Nitrates in foodstuffs have most often been determined by spectrophotometric methods. Formation of coloured products was achieved by the reaction of nitrates with 2-(*sec*-butyl)phenol in acid medium [1], nitration of *p*-cresol [2] or by reactions leading to azo dyes. Nitrites react in acid medium with sulfanilic acid or sulfanilamide and on subsequent coupling with 1-naphthylamino-7-sulfonic acid or *N*-(1-naphthyl)ethylenediamine [3, 4] give red compounds. The intensity of colouration is proportional to the content of NO_2^- ions. Nitrates have to be reduced first to nitrites on cadmium column.

These methods are time-consuming, exacting as to maintaining the same reaction conditions and are applied in the case of deproteinized extracts. The advantage of spectrophotometric methods lies in simple instrumentation.

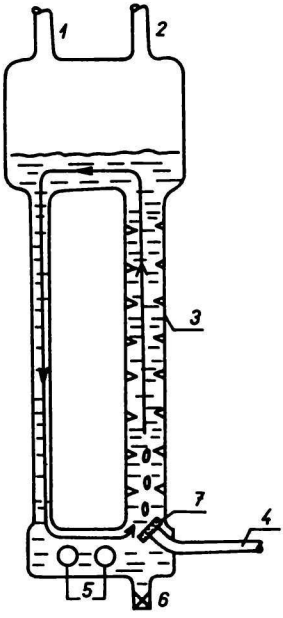

Fig. 1. Absorption apparatus enabling spectrophotometric checking of NH_3 absorption.
1. Outlet for carrier gas; *2.* inlet for titrant; *3.* absorption column; *4.* inlet for gas from the reaction medium; *5.* photoresistors; *6.* outlet gaps; *7.* sintered glass.

For determination of nitrates in waters *Chýlková et al.* [5] published a method based on photometric microtitration. The principle of this method lies in reduction of nitrates in 4 M-NaOH to ammonia which is released from the solution by boiling and carried away by air into an absorption apparatus (Fig. 1). Here it is absorbed in solution of 0.1 M-NH_4Cl containing an acid-base indicator (methyl red). The pH of the absorption packing is adjusted prior to absorption so that the ratio of concentrations of dissociated and undissociated forms of the indicator $[I^-]/[HI]$ is somewhat less than one, *i.e.* the HI form predominates in the mixture (reddish solution). Absorption of ammonia brings about an increase of the pH of the medium, accompanied by colour change (the packing turns yellow). This colour change is followed spectrophotometrically. The bottom part of the absorber is a cell put against the light source. The light passes through the cell and impinges upon two photoresistors which are part of the Wheastone bridge. In front of the photoresistors there are optical filters transmitting those wavelengths of radiation which are maximally absorbed by the individual forms of the indicator. By the resistor divider, forming the second

arm of the bridge, a balance of potentials may be set at the beginning of determination, *i.e.* there is no flow of current through the diagonal of the bridge. At the colour change of the absorber packing the ratio of intensities of radiation impinging upon the photoresistors changes and so does the resistance. This is reflected in unbalanced bridge. The current generated by this change makes function the automatic burette, *i.e.* to dose HCl till the original pH of the packing is restored. The consumption of acid is proportional to the content of nitrates in the sample. The method is absolute and, therefore, suitable for control analyses by ISE electrodes.

With regard to automatic performance of analyses and simple handling of the equipment, it is advantageous to apply this method also in analyses of nitrates in biological materials. The present work deals with elimination of some compounds, namely amino acids and proteins, occurring in biological samples and interfering at the conditions of analyses.

Experimental

Chemicals and solutions

The solutions of 0.01 M-$NaNO_3$, 0.1 M-NH_4Cl, and 4 M-NaOH were prepared by dissolution of salts (anal. grade; Lachema, Brno) and filling up the solution to the defined volume. The 0.1 M-HCl solution was prepared by diluting concentrated HCl. Standardization was carried out in usual way. For reduction powdery Deward alloy (anal. grade; Lachema, Brno) was used. Model solutions were prepared from asparagine and egg protein.

The operational system serving for isotachophoretic determination of nitrates was composed of the leading electrolyte containing 0.008 M-HCl, 0.0015 M β-alanine, 5×10^{-4} M-$CaCl_2$, and 0.05 % poly(vinyl alcohol) and of the terminating electrolyte containing 5×10^{-3} M citric acid.

Equipment and experimental conditions for isolation of nitrates from biological samples

For determination of nitrates the above-mentioned photometric titration [5] was chosen. Application of this method for determination of nitrates in biological material desires elimination of interfering proteins and amino acids present in the sample. For this purpose an electrophoretic separator, enabling fast isolation of NO_3^- ions from the

matrix, was used (Fig. 2). The equipment is composed of three compartments made of transparent organic glass. The individual compartments are separated by cellophane membranes and all parts are screwed up to form a tight unit. The side compartments are equipped with platinum electrodes dipped into the electrolyte, while the central compartment serves for dosing of the sample. The individual compartments are filled with polyethylene syringes through the bottom gaps up to the gaps in the upper part which serve for deaeration of compartments or as outlets for gases formed during electrophoresis. Polyethylene syringes at the same time tighten the whole system.

Separation of ions is achieved in the electric field generated between the electrodes after imposing a certain voltage, provided that the whole system is conductive. Control

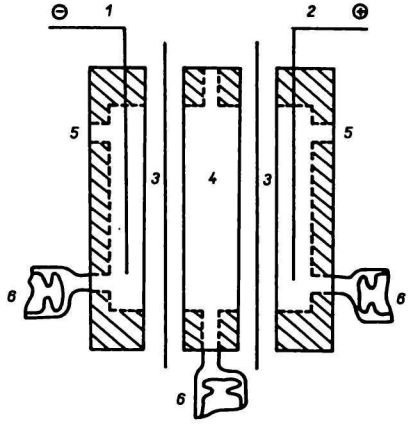

Fig. 2. Scheme of electrophoretic separator.
1. Cathode; *2.* anode; *3.* cellophane membranes; *4.* dosing compartment; *5.* deaeration gaps; *6.* polyethylene syringes.

isotachophoretic analyses were performed with a CS Isotachophoretic Analyser (ÚRVJT, Spišská Nová Ves).

Results and discussion

The migration rate of ions to the respective electrodes in the given medium depends on their mobility and is influenced by intensity of the electric field where the ions migrate. Thus, the motion rate of the particles may be experimentally influenced by change of the intensity of the electric field E. Higher migration rates can be achieved by increasing the intensity of the electric field, *i.e.* by imposing higher voltage. However, at the same time the electric current flowing through the system increases and this leads, in dependence on the resistance of

the medium, to higher heat generation which may cause unwanted boiling of the electrolyte. Total increase of ion concentration in the system leads to the same phenomenon.

Optimum course of separation of NO_3^- ions from the heterogeneous mixture of biological sample with the proposed electrophoretic separator was achieved at the voltage gradient of 6 V cm^{-1} in the electrolyte composed of 0.1 M-Na_2SO_4 and 0.01 M-H_2SO_4.

The procedure proposed for isolation of nitrates from biological samples, eliminating proteins and amino acids which interfere in subsequent photometric determination of nitrates, appears to be sufficiently effective and fast. Total separation of nitrates in the anode compartment takes place within 5 to 10 min, while amino acids present in the sample accept H^+ ions from the acid medium and, as cations, migrate to cathode. The acid electrolyte brings about denaturation of proteins which precipitate and remain in the central dosing compartment. Solutions rich in proteins or samples of pasty consistence obtained on homogenization of the plant material require longer time for separation because the membrane separating the anode compartment becomes evidently clogged up during electrophoresis. It is therefore necessary to clean the membrane after separation. The dosing compartment is filled with redistilled water and voltage is imposed for a short time at reversed polarity of electrodes. The membrane is freed from flocks of organic material and the whole compartment is rinsed with redistilled water. It can be stated further that increased time of electrodialysis (followed for 30 min) does not influence the concentration of NO_3^- in the anode electrolyte.

The efficiency of isolation of interfering components from the samples (proteins and amino acids, reflected in high positive error of determination of NO_3^-) by the proposed method was studied on model solutions of nitrates containing egg protein and asparagine, or with model solutions of carrot juice. Table 1 presents the results of photometric titration of the nitrate samples containing egg protein, obtained after subjecting the samples to preliminary electrophoresis. Analyzed were the anode electrolytes, transferred quantitatively into the apparatus for the determination itself. From the values presented it is obvious that the interference of proteins was eliminated and all NO_3^- ions passed from the sample into the anode compartment.

The experiments performed with carrot juice, were complicated due to different content of nitrates practically in each carrot sample. Therefore, it was necessary to take account of different values of blank experiments in each case. Fig. 3 illustrates the results of determination of nitrates in carrot juice before and after elimination of interfering compounds. It is evident that electrodialysis of samples is necessary, otherwise the results are loaded with high positive error. Already 10 min separation is sufficient for obtaining correct results.

Table 1

Results of determination of nitrates isolated from protein solutions by electrophoretic separation
Electrolyte: 0.1 M-Na_2SO_4 + 0.01 M-H_2SO_4; voltage 12 V; time of separation 5 min

Dose mg NO_3^- / mg egg protein	Found in anode electrolyte $\bar{m}(NO_3^-)$ / mg	Relative standard deviation s_r / %	Interval of reliability for 95 % probability
0.504 59.2	0.488	1.84	0.488 ± 0.01
0.497 75.6	0.496	1.21	0.496 ± 0.007
0.497 92.0	0.490	1.84	0.490 ± 0.01

$\bar{m}$ — average value from five measurements.

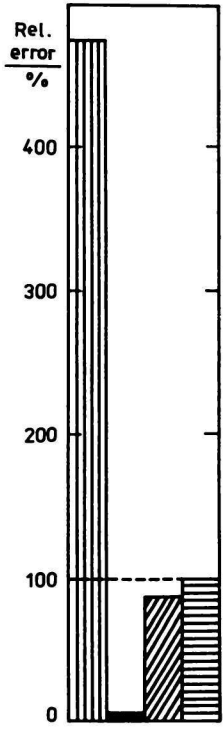

Fig. 3. Schematic illustration of the results of determination of nitrates in carrot juice by photometric titration in dependence on processing of the sample.
Carrot juice without previous separation, carrot juice subjected to electrodialysis for 5 min, carrot juice with standard content of nitrates separated for 5 min, carrot juice with standard content of nitrates separated for 10 min, – – – theoretical value.

The correctness of the found contents of nitrates in unknown carrot juice samples was checked on the basis of comparison with the results obtained by an independent method, *i.e.* isotachophoretic analysis (Table 2). Substantial dif-

Table 2

Comparison of the results of analyses of nitrates in carrot juice samples, obtained by photometric titration [5] after preceding electrophoretic separation and by isotachophoretic technique

Photometric titration $c(NO_3^- + NO_2^-)/(\text{mol dm}^{-3})$	Isotachophoretic analysis		Relative error
	$c(NO_3^-)/(\text{mol dm}^{-3})$	$c(NO_2^-)/(\text{mol dm}^{-3})$	%
6.05×10^{-3}	6.00×10^{-3}	*a*	0.77
2.61×10^{-4}	2.97×10^{-4}	*a*	12.1
2.18×10^{-4}	*a*	*a*	
1.95×10^{-2}	1.64×10^{-2}	4.33×10^{-3}	−5.93
1.55×10^{-2}	6.88×10^{-3}	7.89×10^{-3}	4.94
7.44×10^{-3}	1.76×10^{-3}	5.64×10^{-3}	0.54
9.01×10^{-3}	9.41×10^{-3}	*a*	−4.25

a) Concentration below the determinable limit.

Table 3

Application of the proposed method in analyses of nitrates in chosen carrot samples
Electrolyte: 0.1 M-Na_2SO_4 + 0.01 M-H_2SO_4; voltage 12 V; time of separation 10 min

Content of nitrates in carrot[a]	Content of nitrates[b]
ppm	%
423	84.6
519	104
266	53.2
181	36.2
589	118
574	115
279	55.8
29.8	6.0
2720	544
2030	406
1210	242
1660	332
962	192
67.1	13.4
920	184
884	177
338	67.6
543	109

a) The values given as $NaNO_3$. *b*) The highest admissible values = 100 %.

ference between the found results (higher than 10 %) was observed only in determination of relatively low concentrations of the analyzed ions (approximately 2×10^{-4} mol dm^{-3}). The amount of the NO_3^- ions varying within the limits of permitted standards was found by both methods to be almost the same (the relative error of the obtained results ranged from +5 to −6 %). The NO_3^- and NO_2^- ions may be distinguished in a single run of isotachophoretic analysis. The photometric titration method determines the sum of both ions. The content of nitrates only may be determined after acidifying the sample and removal of HNO_2 by boiling.

Table 3 brings the results of practical application of the suggested method, *i.e.* the contents of nitrates in chosen carrot samples. The samples of carrot juice were prepared by boiling for 2 to 3 min and pressing of the crushed homogenized carrot. The remained debris was dried to constant mass. It is evident from the table that only 38.9 % of the given set of samples met the requirements of the hygienic instruction No. 63 from the year 1986, which prescribes 500 mg $NaNO_3$/kg sample as the highest admissible amount of nitrates in root vegetables. The amounts of nitrates in the remaining samples exceeded the prescribed standard.

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