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Nitrite and nitrate can be accurately measured in samples of vegetal and animal origin using an HPLC-UV/VIS technique

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ABSTRACT

Measurements of nitrite and nitrate are used in biomedical research to estimate the endogenous formation of nitric oxide (an important biomolecule). These anions are also toxins and their concentration is regulated in certain foodstuffs. There are many published methods for detecting nitrite and nitrate but most of them fail to detect nitrite in biological samples. A new HPLC-UV/VIS method was developed which easily detects low concentrations of nitrite and nitrate present in mammal blood, urine and in vegetal samples. The method is based on a pre-column derivatization of nitrite anion using the Griess reaction and direct determination of nitrate using its UV absorbance. A chromatographic process with detection at two wavelengths allows the determination of both anions in one run (23 min with column reequilibration included). The limits of quantification in mammal blood are 2 ng/ml and 200 ng/ml for nitrite and nitrate, respectively. As regards vegetables, due to the need of sample dilution in the preparation steps, these limits are 3 times higher. Concentrations measured in rabbit blood samples ranged from 1.09 to 42.65 μg/ml for nitrate and 15.8 to 384.6 ng/ml for nitrite. Concentrations in vegetables ranged from below the limit of detection to 4 g/kg for nitrate and from below the limit of detection to 369.2 μg/kg for nitrite. The specificity of Griess reaction toward nitrite is under discussion since substances able to mimic this reaction were found, leading to compounds with spectral properties in visible domain indistinguishable from that of nitrite related azo dye.

1. Introduction

Determination of nitrate and nitrite anions in biological matrices is important for two reasons:

a. These anions represent degradation products of nitric oxide – an important biomolecule; the presence of these substances in different types of biological fluids is relevant to the nitric oxide concentration.
b. These anions are toxic substances and their presence is regulated in some types of foodstuffs.

Unfortunately, an easily applicable method for the detection of both nitrite and nitrate anions in biological samples has not been published up to the present time. Nitrite is usually more difficult to detect than nitrate due to the low amounts present in biological material. Nitrite concentrations are about one hundred time lower than those of nitrate, while its toxicity is about the same order of magnitude higher.

Frequently used for simultaneous detection of nitrite and nitrate are the reversed phase HPLC methods based on the use of tetrabutylammonium as an ion pair reagent. The detection is made in UV in the range of 205–220 nm. While perfect for the detection of ppb range of concentrations in very simple matrices [1,2], these methods are ineffective for the detection of low amounts of nitrite in biological samples, because many unretained, highly UV absorptive peaks are covering the small nitrite peak. This problem is not as obvious for the nitrate detection due to its higher concentration and better retention on the HPLC column.

UV detection of these anions can also be made in plasma ultrafiltrates by using capillary electrophoresis [3,4]. Nitrite, however, is not always successfully detected in human plasma and urine by such methods [3].

Ion pair chromatographic methods are successfully used in some cases in foodstuffs with high amounts of nitrite/nitrate (e.g. ham) but there are obtained unsymmetrical peaks or peaks with starting and ending points not well defined on the baseline [5].

Ion chromatography coupled with conductivity detection is also employed in nitrite and nitrate measurements, tens of ppb being the limit of detection. Unfortunately this method is prone to interference of chlorine anion with nitrite, thus lowering its detectability [6,7]. Post column derivatization based on the Griess reaction is

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frequently used for food analysis but limits are no lower than ppm levels [8].

The best way to quantify both anions simultaneously, with high detectability (ppb or even ppt levels), high specificity (use of ^{15}N nitrite and nitrate) and good accuracy is to use a GC–MS [9–14]. Unfortunately, these methods need complicated derivatization procedures that are time-consuming. Other disadvantages of these methods are high costs of infrastructure and reagents.

Other published methods are not usually able to detect both compounds simultaneously or have limited detectability: direct determination in spinach leaves of nitrate using visible-near infrared spectra [15], derivative spectrophotometric determination of nitrate using derivatization to nitrosalicylic acid [16], fluorimetric determination of nitrite using 2,3-diaminonaphtaline [17], etc.

Most often, when determination is made in biological samples, methods rely on the detection of nitrite as a red azo dye, using a spectrophotometer, after derivatization with sulphanilic acid and 1-naphtylamine [18–23]. This reaction is widely accepted to be specific for nitrite anion.

Nitrite is usually present in human and animal plasma in relatively low amounts compared to nitrate anion and its concentration falls under the detection limit. Because of this, in most cases both metabolites of nitric oxide are spectrophotometrically determined as a sum by reducing nitrate to nitrite [19–23].

The goal of this paper was to obtain a simple, sensitive, fast and cost-effective method for the detection of nitrite and nitrate in a variety of biologically derived matrices: blood, urine, and plant material. A simultaneous detection, especially from blood, is considered important since in humans and laboratory animals (mouse, rat, rabbit, and guinea pig) a large amount of nitrate present in the organism comes form alimentary sources. It means that nitrite concentration better describes the formation of nitric oxide than nitrate does.

2. Materials and methods

2.1. Apparatus

HPLC-UV/VIS configuration: quaternary pump Merck Hitachi L-7100, auto sampler Merck Hitachi L-7200, column thermostat Merck Hitachi L-7360, DAD detector Merck Hitachi L-7455, interface Merck Hitachi L-7000, solvent degasser Merck Hitachi L-7612, software D-7000 HSM-Manager, LichroCART 250-4, Lichrospher 100, Rp-18 (5 μm) Merck KgaA Column and LichroCART 4-4, Lichrospher 100, Rp-18 (5 μm) guard column Merck KgaA.

HPLC-VIS–MS configuration: Schimadzu LC: LC 10AD pump, DGA 14A degasser, SCL 10A controller, SPD-M10A UV detector, LCMS-QP 8000 mass detector, X Bridge C18, 2.5 μm, 2.1 mm × 50 mm column (Waters).

2.2. Chromatographic conditions

2.2.1. Chromatographic conditions for HPLC-UV/VIS experiments

Mobile phases: tetrabutylammonium hydroxide 5 mM brought to pH 2.5 with sulfuric acid (A), acetonitrile (B) and methanol (C); the following gradient elution program was used (Table 1):

- injection volume: 100 μl;
- DAD domain 200–600 nm, chromatograms were extracted at 222 nm and 520 nm;
- peak purity limit: 98%;
- analysis time: 23 min.

Table 1
Gradient used in the HPLC-UV/VIS experiment.

Time (min)	A (%)	B (%)	C (%)	Flow (ml/min)
0	92	8	0	1.000
8.5	92	8	0	1.000
10.5	48	8	44	1.000
15.5	48	8	44	1.000
15.6	92	8	0	1.000
18.5	92	8	0	1.000
18.6	92	8	0	1.800
22.0	92	8	0	1.800
23.0	92	8	0	1.000

Table 2
Gradient used in the HPLC-UV–MS experiment.

Time (min)	A′ (%)	B′ (%)	Flow (ml/min)
0	80	20	0.200
20	80	20	0.200
25	20	80	0.200
25.1	80	20	0.200
30	80	20	0.200

2.2.2. Chromatographic conditions for HPLC-UV/VIS–MS experiments

Mobile phases: 1% acetic acid in purified water (A′), 1% acetic acid in methanol (B′); the following gradient elution program was used (Table 2):

- injection volume: 20 μl;
- UV/VIS detector settings: DAD range: 200–700 nm; extracted chromatograms: 220, 400, 450, 500, 550 nm;
- MS detector settings: ESI ionization, *m*/*z* interval: 50–500; acquisition mode: scan; polarity: positive; detector gain: 2.7 kV; probe high voltage: 5 kV; nebulizer gas flow: 4.5 l/min; CDL temperature: 250 °C; CDL voltage: 10 V; deflector voltages (all): 70 V.

2.3. Reagents

Sulphanilic acid (p.a. ≥99.0%) Fluka Analytical, 1-naphtylamine analytical standard Fluka Analytical, Acetic acid 100% Merck KGaA, tetrabutylammonium hydroxide 20% Merck, methanol gradient grade for HPLC Merck KGaA, acetonitrile gradient grade for HPLC Merck KGaA, sodium nitrite p.a. Merck KGaA, sodium nitrate p.a. Merck KGaA, purified water for HPLC.

Griess A reagent: 50 mg sulphanilic acid was dissolved with the use of ultrasound in a mixture consisting of 1.5 ml acetic acid and 3 ml purified water.

Griess B reagent: 4 mg 1-naphtylamine was dissolved in 4 ml acetic acid.

After preparation Griess A and Griess B solutions were kept in a refrigerator and were usable for 3 days.

2.4. Sample treatment and calibration curve

2.4.1. Rabbit blood

Eight New Zeeland white rabbits (blanks and subjected to different nitric oxide donor treatments) were used for blood testing. Animal experiments were made by respecting the international laws and with permission obtained from our University's Commission on Ethics.

1 ml of venous blood was sampled in anticoagulant free 3 ml polyethylene tubes. It was centrifuged 10 min at 3500 rpm as soon as possible. To 100 μl of serum 100 μl of acetonitrile was added for deproteinization, followed by centrifugation (10 min at 14,000 rpm). 150 μl supernatant was mixed in an HPLC vial with 150 μl mobile phase A and 30 μl Griess A reagent. After 1 min, 30 μl

Griess B reagent was added and the solution was mixed again. The injection can be made anytime between 0.5 and 48 h after finishing the sample preparation steps.

2.4.2. Human, rabbit and rat urine

Same sample treatment as in the case of blood but without the first centrifugation step.

2.4.3. Vegetables

The juice resulted after grinding the plant material was frozen (−20 °C) until analysis. 500 μl defrost juice was mixed with 1 ml water and 1.5 ml acetonitrile, and then the mixture was centrifuged at 12,000 rpm. 500 μl of the supernatant was mixed with 500 μl of mobile phase A and 100 μl of Griess A reagent. After 1 min 100 μl of Griess B reagent was added. The solution can be injected into the HPLC system any time between 0.5 and 48 h.

In the case of vegetables' nitrate concentrations, they sometimes exceeded the highest value in the calibration curve. In this case, the sample (the juice obtained by grinding the plant material) was diluted and reanalyzed.

2.4.4. The calibration curve

The calibration curve was obtained by injecting into the HPLC system solutions with known amounts of nitrate (0.2–200 mg/l) and nitrite (6–400 μg/l) that were previously treated as the blood samples. No centrifugation was applied for standard solutions.

3. Theory

As mentioned in Section 1 measurements of nitrate and nitrite anions in various matrices are made with difficulties, especially due to the fact that nitrite is prone to fall under the detection limits of most methods used. That is why for biomedical researches, as general rule, the sum of nitrite and nitrate is measured after an enzymatic nitrate reduction. Nitrite determination is very important for such researches because it describes better nitric oxide generation opposed to the sum of nitrite and nitrate.

We previously published an HPLC-VIS method for the detection of nitrite in complex UV absorptive matrices [24]. The method relies on the pre-column derivatization of nitrite with sulphanilic acid and 1-naphtylamine when a red azo dye is formed. The limit of quantification is 5 ng/ml (same order of magnitude as in the case of results published for some GC–MS methods) and the run time is of only 6 min. Because the readings are made at 520 nm the baseline is extremely clear. The only disadvantage was the impossibility of determining nitrate in the same run, too. The determination of nitrate could be done by reducing it to nitrite and reanalyzing the sample. Many low cost, non-enzymatic methods described in the literature for nitrate reduction to nitrite were tried without success.

HPLC-UV ion-pair methods, that were also tried, allowed the determination of biologically relevant nitrate concentrations (limits of quantifications were usually, hundreds of ng/ml), but were useless for nitrite measurements. Fig. 1a shows a chromatogram of a urine sample obtained using a HPLC-UV method, described elsewhere [25], comparable with other recently described methods [2,5]. The improvement brought by the method [25] over the ones that already existed is the improved baseline of the standard solutions (Fig. 1b). As it can be seen in Fig. 1a nitrite could not be detected or measured in any way due to the intense baseline changes caused by UV absorbing substances, even if detectability for water samples is astonishing (Fig. 1b). It is not possible to make a chromatographic separation from the large number of hydrophilic substances also present in urine because nitrite has a relatively low retention on the chromatographic column when RP-ion pair

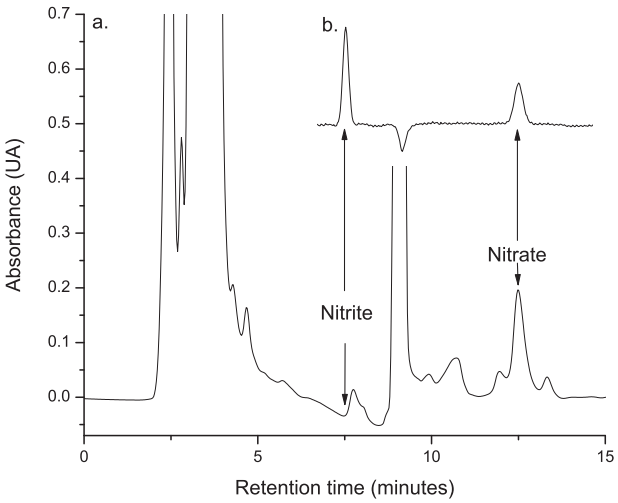

Fig. 1. (a) Chromatogram of a urine sample, containing 24 ng/ml nitrite and 15.2 μg/ml nitrate, obtained by an HPLC ion-pair method [25]; (b) Chromatogram of a standard solution (70 ng/ml nitrite and 70 ng/ml nitrate) using the method previously mentioned.

methods are used. Nitrate, a better retained compound, could be detected by this method. A better separation from the neighboring peaks is possible by making pH changes.

Because of these considerations, it is clear that a combination of the two methods would be a promising perspective for both anions' simultaneous detection. Lower sensitivity for nitrate is not a problem since it has a far lower toxicological and biomedical importance than nitrite has and is present in high amounts in biological samples. However, ion pair chromatography with UV detection has a detectability for nitrate that is not overcome by other usually employed methods.

The combination of the methods is easy because nitrate is not affected by the Griess reagents' components and the azo dye resulted from nitrite gives a better shaped peak on the HPLC system if the ion pair reagent – tetrabutylammonium – is present. Detection is to be made in UV for nitrate and in visible domain for the nitrite derived azo dye, but the analysis can be made in one run using a DAD or a two channel UV detector.

The proposed HPLC-UV/VIS method is able to detect nitrite without derivatization by working as a simple HPLC reversed phase

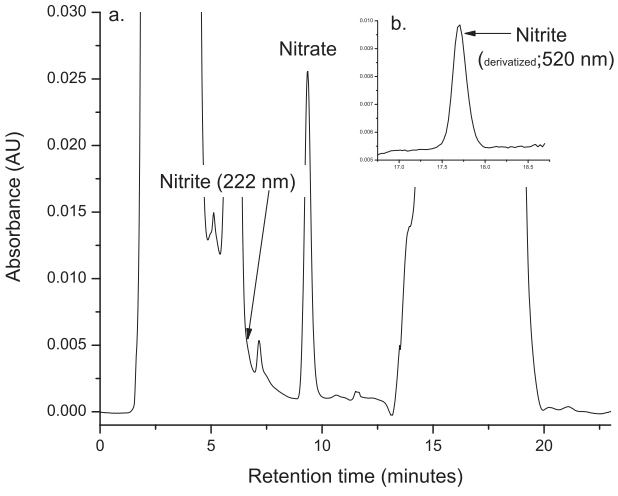

Fig. 2. (a) Chromatogram of a human blood sample containing 7.49 μg/ml nitrate and 74.2 ng/ml nitrite obtained without derivatization (222 nm); (b) Chromatogram obtained for nitrite ion after the derivatization process (520 nm).

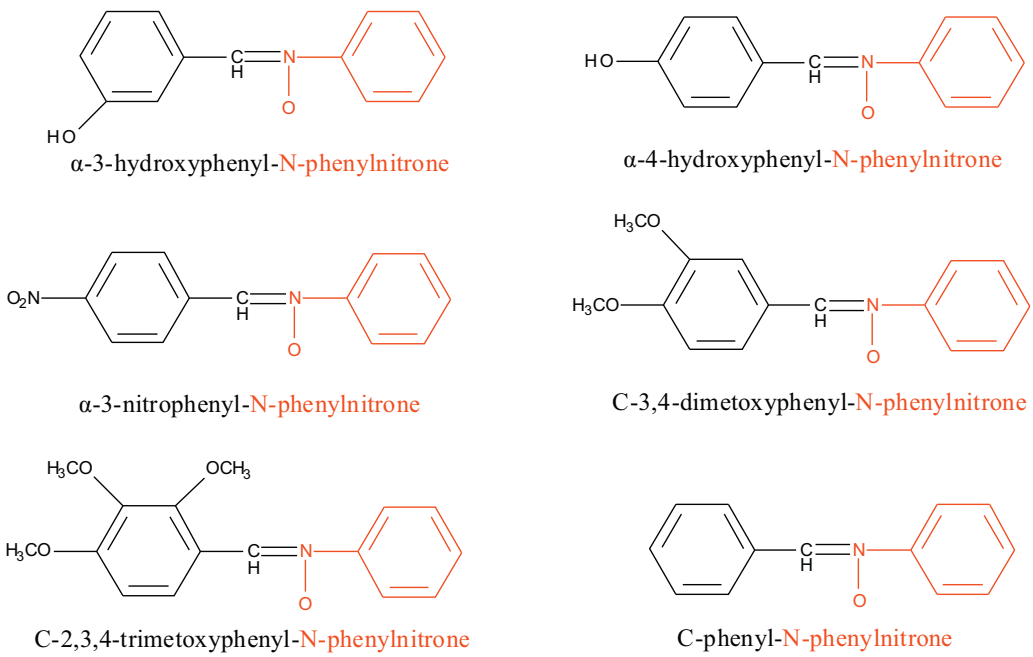

Fig. 3. Structure of compounds found to interfere with the Griess reaction.

ion-pair method, but in biological samples the derivatization process is absolutely necessary due to the low amount of nitrite, its low retention and the UV absorption of the interfering agents. Fig. 2 shows a chromatogram of a blood sample where nitrite is undetectable without the derivatization step (Fig. 2a) but is perfectly measurable after the derivatization process (Fig. 2b). The same conclusion was drawn for urine and vegetal samples.

4. Results and discussion

4.1. Specificity issues regarding the Griess reaction

The Griess reaction was considered specific to nitrite anion for more than 150 years. During a previous research on substances with ability to release nitric oxide it was observed that a class of chemicals: N,α-diphenylnitrones (structures presented in Fig. 3) have the ability to mimic the Griess reaction under certain conditions (decomposition induced by UV irradiation) without the presence of spectrophotometrically measurable nitrite concentrations.

When Griess A and B reagents were added to the UV irradiated solution of nitrones, it was obtained a red color similar to that obtained for nitrite.

HPLC-VIS–MS experiments showed that there were at least 2 colored compounds (A and B in Fig. 4) with retention times and MS spectra different to that of the commonly obtained nitrite related azo dye. These colored compounds that mimicked the Griess reaction do not appear in the freshly prepared nitrones solutions, nor in the solutions left for more than 2–4 h under the UV light. This means that an intermediate decomposition product of the nitrones is responsible for the reaction. Retention times and MS spectra of the colored compounds showed that these compounds are common among the five available nitrones. One can assume that the N-phenyl fraction of the molecule is responsible for the reaction since the C-phenyl fraction was substituted with different well bound substituents in different positions. The ESI-MS spectra of the main colored compound B (RT 17.9 min) showed a parent compound with an m/z of 325 and a fragment with an m/z of 222.

Until the structure of these compounds and the chemical reaction through which they are formed are elucidated, it is impossible to determine whether a drug or other compound containing a nitrogen atom bound to a phenyl ring mimics the Griess reaction.

Interestingly, the standard Griess reaction was not hindered by the fake one and the standard azo dye, identified by VIS spectra and MS spectra, was formed too in the presence of nitrite (Fig. 4). It means that the Griess reaction is still usable, but in order to make readings in visible domain a chromatographic separation is needed.

4.2. Method performance check

A typical chromatogram obtained for nitrate (RT 9.28) from a vegetal sample is shown in Fig. 5. Chromatograms obtained from samples of animal origin had similar baselines.

Fig. 6 shows a typical chromatogram for nitrite (RT 17.4) from the same vegetal sample the Fig. 5 was obtained. The baseline distortion visible between 12 and 16 min is gradient related and in no way interferes with the detection of very small nitrite peaks. In the case of nitrite, shapes of baseline were identical for vegetal, animal or standard samples.

The red colored vegetal samples were the exceptions, where extra peaks appeared, but they were perfectly separated from the nitrite peak (Fig. 7).

4.2.1. Specificity

Because almost every sample type for which the method was developed (animal blood, urine, vegetables) contains nitrite and nitrate anions it is quite difficult to obtain blanks for these kinds of samples. The use of only the retention time as peak identification tool was not considered sufficient. In order to prove the identity of nitrite and nitrate the following tests were used:

4.2.1.1. Nitrate anion. A nitrate reduction reaction was applied for all types of samples used to see if the chromatographic peak, with a retention time similar to that of nitrate, behaves in the way that a nitrate standard does. Following hydrazine reduction an about 90% peak height reduction was obtained in the tested samples. The same value was obtained when standard solutions were subjected to the same treatment.

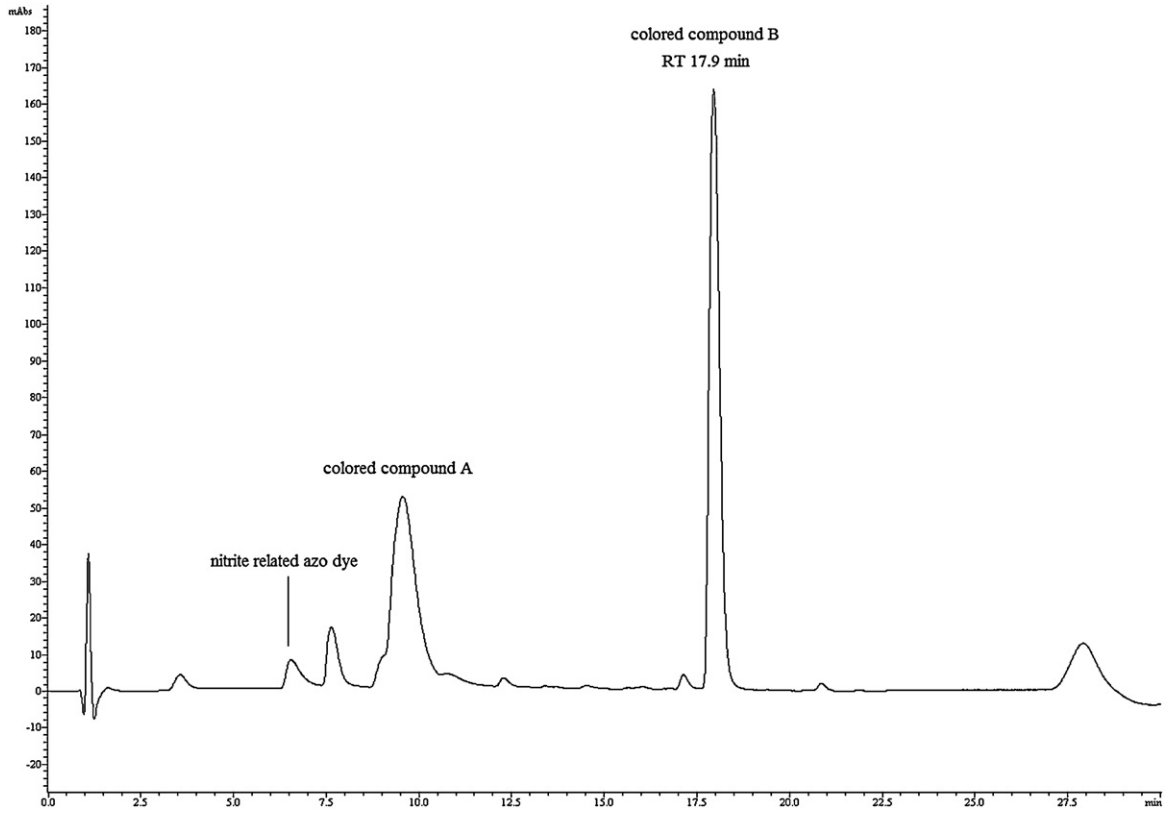

Fig. 4. Visible domain chromatogram (500 nm) obtained for C-phenyl-N-phenylnitrone UV irradiated for 1 h and then treated with Griess A and B reagents.

Furthermore, peak purity was assessed using the DAD detector on the 200–600 nm range finding that all peak purities were higher than 98%. Because of the specific UV absorption spectra of nitrate (a very narrow absorption spectra with a maximum at 216 nm) one can consider peak purity a good measure for the specificity of the method. Certainly, other nitroderivates can have the same absorption spectra as nitrate, but these compounds are not present in relevant amounts in biological samples, and their lipophilicity ensures higher retention time than that corresponding to nitrate.

In order to further increase specificity for the nitrate anion, second derivatives of the peaks were computed in every type of sample and they were compared with second derivatives recorded in standards.

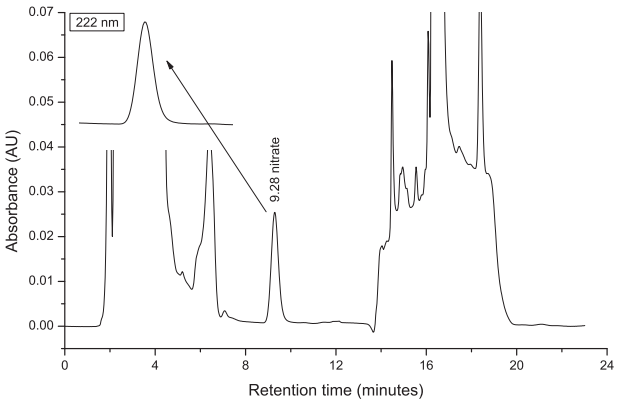

Fig. 5. A typical chromatogram of a vegetable sample (nitrate concentration: 25 mg/kg, detection wavelength: 222 nm).

4.2.1.2. Nitrite anion. The derivatization process applied in the case of nitrite is also a possibility to obtain blanks for samples that contain this anion and to see if any biological constituent is interfering with the analyte. Obtaining such blanks is of high importance for nitrite because it is present in far lower amounts than nitrate and any small amount of interfering compounds can significantly alter the results. No peak with retention time similar to that of the nitrite-related azo dye appeared on chromatograms when water, samples lacking one of the Griess reaction constituents, or Griess reaction constituents were injected.

However, such peak usually appeared when a mixture of Griess A and Griess B reagents was injected. The resulted peak had a small height, usually 10 times higher than the baseline noise. Because the peak appeared only when Griess A and Griess B reagents were mixed, it was linked with the presence of extremely low levels of nitrite in the reagents used for sample treatment or for the derivatization process (approximate 0.2–0.3 ng/ml; an estimation made using the information on the label of the reagent bottle). Another source of nitrite in the samples is the dissolution of nitrogen oxides (existing as normal constituents or pollutants in the air). This sample contamination process is described in the scientific literature and is observed when nitrite is measured using methods with high detectability [13,24]. The contamination of blanks can be avoided if preparation is made in a nitrogen oxides free environment (a glass box in which air is introduced after being passed through a sodium hydroxide solution). In this case perfect blanks are obtained without any interferences, and is obvious that nitrogen oxides dissolved from the air are the main reasons for the sample contamination.

Working in nitrogen oxides free environment is unfeasible due to the many processes involved in nitrate and nitrite analysis (like drawing blood from animals or humans, centrifugation, reagent

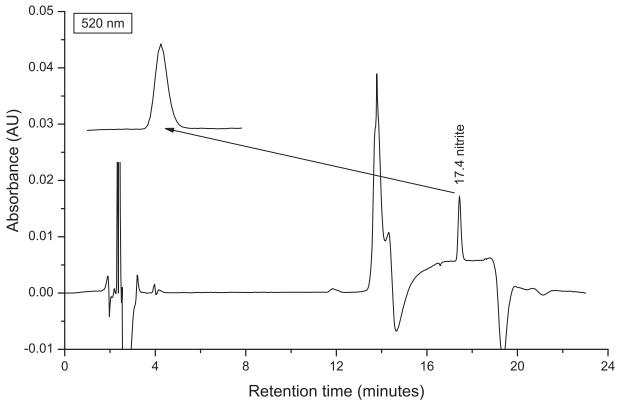

Fig. 6. A typical chromatogram of a vegetable sample (nitrite concentration: 41.6 μg/kg, detection wavelength: 520 nm).

preparation, sample treatment, etc.). In order to remove the effect of contamination it was decided that blanks ($N = 5$) have to be treated and analyzed exactly as the samples. The average peak area recorded for the blanks should be subtracted from the areas recorded for the samples. It is also important to note that the area of the nitrite peak resulted from contamination is in most cases far lower than 5% of the area resulted in samples after blank subtraction. It means that this type of contamination does not hinder the determinations made in biological samples. Such a sample contamination can influence the results only when the lowest limit of detection is to be determined or very low nitrite content samples are analyzed.

Hemolyzed blood samples or the red color of fruits and vegetables, where existed, were not a hindrance in the red azo dye determination because the column separation ensured the lack of interferences. Even beetroot samples, intensively colored in red, permitted the analysis of low amounts of nitrite without any interference (Fig. 7).

4.2.2. Linearity

4.2.2.1. Nitrate anion. Linearity was evaluated for a concentration range of 0.2–200 mg/l (7 points). Intraday linearity was tested by analyzing 5 sets of calibration standards (slope $29{,}271 \pm 98$ μV s and intercept $-1.7 \times 10^{-3} \pm 1.7 \times 10^{-3}$ μV s). The coefficient of correlation ($R = 0.99996$) and residuals with values lower than 10% (residuals do not correlate with concentration) showed a good linear response between concentration and the peak area. The coefficients of variation, calculated for all points of the calibration curve, did not exceed the 2.5% value. Interday linearity was tested by analyzing three sets of calibration standards in three consecutive days. Results were similar to those recorded for the intraday linearity.

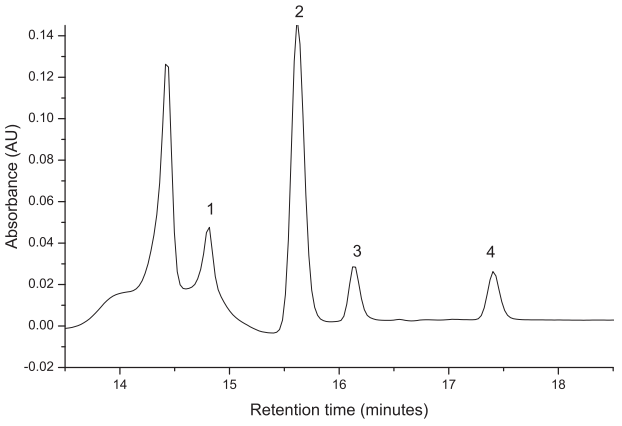

Fig. 7. The chromatogram of the nitrite derived azo dye obtained for a beetroot sample (1–3 beetroot derived peaks, 4 nitrite derived azo dye peak).

4.2.2.2. Nitrite anion. Linearity was evaluated for a concentration range of 6–400 μg/l (5 points). Intraday linearity was tested by analyzing 5 sets of calibration standards (slope 366.9 ± 1.4 μV s and intercept $-0.15 \times 10^{-3} \pm 0.27 \times 10^{-3}$ μV s). The coefficient of correlation ($R = 0.99994$), residuals (less than 10%), and coefficients of variation (less than 3.5%) showed a good linear response between the peak area and concentration.

4.2.3. Accuracy and precision

In order to obtain information about accuracy and precision of the method the spiked samples technique was used. The content of nitrate and nitrite present in samples before spiking was subtracted from the final values.

4.2.3.1. Rabbit plasma. Tubes containing anticoagulant were tested and found positive for nitrite. Due to this fact, it was important to use coagulant free tubes in order to reduce sample contamination. The drawback of the anticoagulant free tubes was the impossibility of spiking blood due to the quick formation of a blood cloth after the sampling procedure. Because of this consideration, accuracy and precision estimation were made using serum instead of blood.

Serum from 2 rabbits was spiked with nitrate and nitrite to form the final theoretical concentrations of nitrite: 60, 150 and 300 ng/ml and nitrate: 6, 15, 30 μg/ml ($N = 5$ for each concentration). The accuracy was 99.09–103.80% for nitrite and 96.78–102.20% for nitrate. All coefficients of variation were below 2.5%.

4.2.3.2. Plant materials. Potato, onion and apple samples (5 for every type of product tested) were grinded and the obtained juice was sampled with nitrate (3, 10, 30, 100 mg/l) and nitrite (30, 60, 150 and 300 μg/l). The initial concentrations present in samples were subtracted from the results. The recovery ranged between 97.20 and 103.55% for nitrate and 98.29 and 104.05% for nitrite. The coefficients of variations recorded for every concentration level did not exceed 5%.

4.2.4. Limits of detection and quantification

For rabbit and human plasma the limits of detection (concentration of analyte yielding a signal to noise ratio of 3:1) and quantification (concentration of analyte yielding a signal to noise ratio of 10:1) for nitrate ion are 0.06 and 0.20 μg/ml (mg/l), respectively. In the case of vegetables, the juice obtained after grinding does not mix in most of the cases with acetonitrile, making the deproteinization process and removal of the acetonitrile-insoluble components impossible. A dilution in a 1:3 ratio with purified water solves this problem but the limits of detection and quantification increase to 0.2 mg/kg and 0.6 mg/kg, respectively.

In the case of nitrite because of the presence of very small but detectable amounts of nitrite in the blanks (due to contamination of reagents or dissolution from air) it is difficult to apply the signal to noise ratio of 3:1 and 10:1 when determining the limits of detection or quantification. Due to this naturally occurring contamination process, a limit of detection cannot be adequately determined. Limit of quantification for solutions of nitrite dissolved in water is 2 ng/ml. At this level of concentration a blank subtracted chromatogram always yield a signal to noise ration of at least 10:1, and the coefficient of variation ($N = 5$) is lower than 10%.

Limit of quantification in serum cannot be exactly determined due to the fact that is impossible to find rabbit blood (or other mammal blood) without nitrite. Because water and plasma lead to similarly baselines in the proximity of the nitrite peak, one can assume a 2 ng/ml limit of quantification for blood, too. Unfortunately, this cannot be experimentally verified until one find a blood sample that contains an undetectable amount of nitrite. The exact value of the quantification limit is not very important for biomedical studies since the lowest recorded concentration of nitrite in blood was 15 ng/ml which is significantly higher that the expected limit of quantification.

Limit of quantification for nitrite in vegetable juices is 6 ng/ml. This lower detectability for nitrite is due to the dilution step in sample preparation and not to interferences from matrix constituents.

4.2.5. The stability of nitrite and nitrate anions in the conditions of the experiment

Nitrite anion is unstable and theoretically can be converted into nitrate after the blood sampling process and during the sample preparation steps. In order to evaluate the extent to which this process takes place, nitrite values obtained with the procedure described in Section 2.4 were compared with those obtained when acetonitrile was added to blood immediately after the sampling procedure. No difference was observed between nitrite concentrations, meaning that nitrite concentrations are stable during sampling and sample preparation procedures. Furthermore, serum samples were left 5–180 min at room temperature. No changes in nitrite concentrations were recorded. Nitrate anion, as expected, was also stable under these conditions.

The use of plain polyethylene tubes for blood sampling is recommended because in some types of tubes containing anticoagulant, nitrite in high enough amounts to interfere with the determination, was found.

No changes in the concentrations of nitrite and nitrate were seen when samples were frozen (−20 °C) for three months.

Stability problems can also be raised by the azo dye formed during the derivatization of nitrite. Its concentration can decrease during the period in which samples are kept in autosampler prior to analysis. The azo dye proved to be stable for at least 48 h if the nitrite concentration in the sample does not exceed 2000 μg/l. If this limit is exceeded a precipitation of the azo dye occurs. All nitrite concentrations were well below this limit and samples were analyzed maximum at 24 h after defrosting and derivatization.

4.2.6. Interference of biological material with the Griess reaction

Section 4.1 shows that certain types of chemicals can interfere with the Griess reaction leading to false readings if a spectrophotometer is used. By using an HPLC separation this inconvenience can be overcome.

It is reasonable to presume that there will also be substances that will consume one or both components of the Griess reagent, or that will interfere with the reaction steps leading to false negative results. During the development stage of this work such interference was observed when trichloroacetic acid which was tried for sample deproteinization. Adding this reagent prior to the derivatization process leads to the complete disappearance of the nitrite related azo dye peak from the chromatogram. No reasonable explanation was found for such strange behavior since another organic acid – acetic acid – is used in both Griess A and Griess B reagents as a solvent (the acidity difference is not the cause, since the Griess reaction takes place well, in the presence of hydrochloric acid too).

In order to avoid such problems, after the HPLC analysis took place 100 μl of a 2000 ng/ml nitrite solution was added to each HPLC vial and the formation of a visible red color was verified. No interference with the formation of the red azo dye was observed during the analysis of about 5–600 biological samples of animal and vegetal origin.

4.3. Uses of the new method

The new method successfully combined the detection of nitrite as an azo dye, having an astonishing detectability, with the simple HPLC-UV detection of nitrate. Sample preparation is kept to a minimum and the cost of the analysis is low. The detection of nitrite is made in visible domain (520 nm) while that of nitrate in UV domain (222 nm). It is worth to mention that nitrate has a narrow absorption maximum at 216 nm but it is still better determined at 222 nm where the peak height is about half of that obtained at 216 nm, but the baseline improvement outweighs this inconvenience. This principle is applied by other authors too [1,2]. In previous works we showed that there are other cases where advantages of readings in other spectral regions than the maximum of absorption could be obtained [26].

Using the new method, it was possible to analyze without interference nitrite and nitrate amounts in animal blood and urine during experiments regarding the ability of some substances to release nitric oxide (data not published). At least 300 samples of rabbit blood were analyzed and no interference was found and no sample was “below the limit of quantification”. The concentrations of nitrate varied between 1.09 and 42.65 μg/ml and those of nitrite between 15.8 and 384.6 ng/ml in blood samples.

The newly developed method was also used for the determination of nitrite and nitrate content of vegetables and soil. Following vegetables and fruits were tested: white potatoes, red potatoes, red onion, white onion, garlic, beetroot, carrot, parsley, celery, parsnip, tomatoes, cucumbers, cabbage, red cabbage, lettuce, radish, different types of apples, oranges, kiwi, strawberries, lemon, and tangerine. No interferences were recorded and only about 5% of the samples fall under the detection limits. However, the detection limits are sensitive enough to detect concentrations of nitrite and nitrate in vegetables that are about 2 orders of magnitude lower than those accepted for drinking water. This means that everything that falls under the detection limit of this method is completely non significant from a toxicological point of view. The concentrations of nitrate recorded in about 200 samples of vegetal origin ranged from below limit of detection to as high as 4 g/kg (lettuce). In the same samples nitrite ranged from below the limit of detection to 369.2 μg/kg (lettuce). The lowest recorded concentrations of nitrate were found in fruit, tomatoes and cucumber samples and the highest in lettuce and beetroot samples. No nitrite concentrations of toxicological importance were found in any of the tested samples.

Compared with other recently published methods based on the Griess reaction [27,28], better detection limits are attained and the sample preparation is kept to a minimum. Even the European Committee for Standardization’s method based on an anion exchange column followed by Griess reaction derivatization for determining nitrate, yields only a working range of 50–3000 mg/kg [28]; no nitrite can be determined by this method. Removing the carcinogenic cadmium from the method is another advantage. When compared with HPLC-UV ion pair methods, one can see that the new method has about 1–3 orders of magnitude higher sensitivity for nitrite when determining it from more complex matrices than water [1]. Usually, almost all other types of methods published so far have by 2–3 orders of magnitude higher detection limits for nitrite in vegetables than the new method (exception GC–MS methods). So far most attempts, published in the scientific literature, to detect nitrite in plant material were without success because almost all concentrations fell under the limit of detection.

5. Conclusions

This paper presents an HPLC-UV/VIS method usable for simultaneously determining nitrate and nitrite in materials of both animal and vegetal origin with high sensitivity, accuracy and precision. Such method is extremely important in the biomedical research regarding the formation of nitric oxide and in the toxicological research regarding the presence of nitrite and nitrate as toxins in vegetables or biological material.

The results show that there are substances able to mimic the Griess reaction, so it can no longer be considered absolutely specific for nitrite anion and spectrophotometric measurements are to be employed with care. Mass spectral data showed that interference is due to formation of other substances than the expected azo dye so a chromatographic separation will solve problems raised by such interferences.

Another advantage of the presented method is the ability to work with samples of different origins without the need of modifications.

The use of easily available equipments, low cost, easy sample preparation and better performance than the other published methods (excepting GC methods for performances as detection limit and specificity) recommend the new method for wide use in various fields where knowing nitrite and nitrate amounts is required.

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