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Rapid and sensitive determination of nitrite in foods and biological materials by flow injection or high-performance liquid chromatography with chemiluminescence detection

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Abstract

A method is described for the determination of nitrite that is based on reduction of nitrite with potassium iodide followed by chemiluminescence detection of the liberated NO using a thermal energy analyzer. The method worked well both in the flow injection mode and upon interfacing with a reversed-phase high-performance liquid chromatography system. Limited data available indicated a good agreement between results obtained by the chemiluminescence method and those obtained by using a well established colorimetric procedure when they were applied for the determination of nitrite in a number of cured meats, human saliva, and baby foods. The chemiluminescence method is, however, much superior to the colorimetric one in its speed, versatility, as well as sensitivity (200 times more), and requires only a minimum of sample preparation. The detection limit of the new method is about 0.1 ng $NaNO_2$ per injection or 5 μg/kg in cured meats and other substrates analyzed.

1. Introduction

There is extensive interest in the determination of nitrite in both foods and biological materials. This is mainly because of three reasons. First, it is highly toxic to man, especially to infants, causing methemoglobinemia and possibly death [1–3]. Secondly, nitrite can lead to the formation of various carcinogenic N-nitroso compounds after reaction with secondary amines and amides which are abundant in foods [4,5]. Thirdly, nitrite is widely used as a preservative in cured meat products and certain kinds of fish that suggests the need for accurate control and monitoring of nitrite levels in such products throughout various stages of processing and storage [2,6]. Nitrite also occurs naturally, mostly at low ppm (μg/g) levels, in water, certain vegetables, storage-abused leafy vegetables (levels can exceed 100 ppm), and many processed foods (*e.g.* cereals and dairy products) [2,7].

Besides ingested nitrite through foods, salivary nitrite, produced due to microbial reduction of nitrate in the oral cavity, constitutes one of the major sources of man's exposure to this chemical [2,8]. There is conclusive evidence to suggest that both ingested and salivary nitrite can react *in vivo* with various amines and amides in the food to form N-nitroso compounds [2,4,9]. Such a reaction can occur in the acidic environment of the human stomach or other parts of the body. This has prompted many to investigate the effects of various nitrate-rich diets on salivary

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nitrite levels and, ultimately, on *in vivo* N-nitrosation [2,4,10].

Numerous methods have been reported for the determination of nitrite in foods and biological materials [11,12]. These include the classical colorimetric methods based on Griess reaction [11,13], polarographic methods [14], gas chromatographic methods following derivatization [15], high-performance liquid chromatography (HPLC) using different detectors [16–21], and chemiluminescence detection following reduction of nitrite to NO using suitable reductants [22–26]. Although these methods are reliable and adequate for the purposes developed, some of them are lengthy and time consuming, some lack specificity or sensitivity, and some have not been tested adequately to determine their applicability to a wide variety of substrates. It was felt that there is a need for a highly sensitive and specific method for the determination of nitrite in foods and biological materials that is fast and requires only a minimum of sample preparation. In this paper, we wish to report such a method that is based on chemiluminescence detection of nitrite after reduction with iodide ion. It requires only a few minutes to 1.5 h to complete an analysis depending on the nature of the sample being analyzed. The determination can be carried out either in the flow injection mode or HPLC mode using a reversed-phase and ion-pair chromatography system.

2. Experimental

2.1. Apparatus

A schematic diagram of the whole detection system used is shown in Fig. 1. It can be roughly divided into three parts: (a) HPLC assembly, (b) post-column reactor for reduction of nitrite to NO, and (c) chemiluminescence detection of NO using a thermal energy analyzer (TEA).

(a) HPLC unit

The unit (Fig. 1) consisted of a solvent delivery system (pump Model 110B, Beckman, San Ramon, CA, USA) and an Altex Rheodyne injector (Model 7125, sample loop 50 μl or 100 μl). The HPLC separation was carried out using a 250 mm × 4.6 mm I.D. stainless-steel column packed with C_{18} packing (5 μm) (Techsil, Cheshire, UK) and a mobile phase consisting of 0.05 *M* KH_2PO_4 (pH 6) and 5 m*M* tetrabutylammonium hydrogen sulfate. The mobile phase flow-rate was either 1 or 2 ml/min. In the flow injection mode, the HPLC column was omitted from the system.

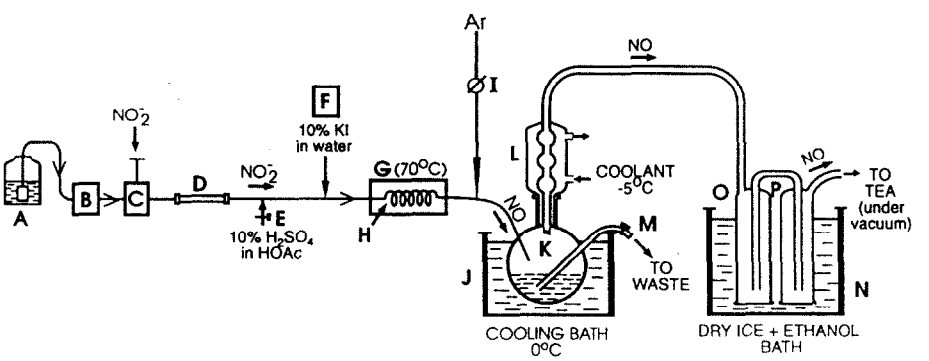

Fig. 1. Schematic diagram of the detection system. (A) Mobile phase; (B) mobile phase delivery system (pump); (C) injector; (D) HPLC column; (E) PTFE stopcock [27]; (F) peristaltic pump; (G) temperature-controlled hot water bath; (H) coiled tubing (reactor); (I) precision gas flow controller; (J) cooling bath (ice + water); (K) collection flask (500 ml); (L) condenser; (M) glass stopcock; (N) cooling bath (dry ice + ethanol); (O) and (P), cold traps. The trap O should be connected exactly as shown so that the moisture collects on the outside wall of the trap. Modified from that described by Havery [27].

(b) Post-column reactor

The reactor used was essentially the same reported previously by Havery [27] for the determination of N-nitroso compounds. The main differences were that, in this study, a temperature-controlled hot water bath (instead of a heating mantle) was used to heat the coiled PTFE tubing (300 cm × 0.16 cm O.D., and 0.08 cm I.D.), and a peristaltic pump was used to introduce the 10% KI solution (flow-rate *ca.* 0.5 ml/min). Also, a condenser was added between the two cooling baths (Fig. 1) for a more efficient removal of moisture that otherwise resulted in frozen lines in traps O or P. It should be emphasized that all tubings and connections that come in contact with NO must be made of PTFE or glass. Further details regarding design and assembly of the reactor can be obtained from the above reference.

As will be discussed later, two other variations of nitrite reduction conditions were investigated. These were: (i) glacial acetic acid alone (instead of 10% H_2SO_4 in HOAc) and no KI solution in flask F, and (ii) 10% ascorbic acid solution in flask F (instead of KI solution) and glacial acetic acid (without H_2SO_4). It should be noted that there was no need to pump in the acid (HOAc or mixture of H_2SO_4/HOAc). It was slowly drawn in by the TEA vacuum which was maintained between 1–1.2 mmHg. Other conditions were as follows: acid flow-rate, *ca.* 2 ml/min; ascorbic acid solution flow-rate, *ca.* 0.5 ml/min; and Ar carrier gas flow-rate, 30–60 ml/min (optimum flow determined by trial and error).

After a few runs, when the flask K became *ca.* 60% full, the TEA vacuum was switched to the vent position which blocked the tubing going into the TEA (thus disconnecting the system from the vacuum). Immediately following this the stopcock E was closed and that at M was opened. Within a few minutes the carrier gas (Ar) pushed out the liquid in flask K into a waste reservoir which should be kept inside a fume-hood. After emptying the flask the above manipulations were carried out in the reverse order and analyses were resumed after a few minutes of equilibration. Care should be taken to watch for a frozen line in the system that is usually indicated by a sudden drop in TEA vacuum or by a build-up of pressure inside flask K thus resulting in a reverse flow of KI solution into the flask containing the H_2SO_4/HOAc mixture.

(c) Chemiluminescence detection

The NO produced by reduction of nitrite and purged out by the carrier gas was detected using a TEA detector (Model 502, Thermedics Detection, Chelmsford, MA, USA). Basically, it is a chemiluminescence detector originally developed for specific determination of N-nitroso compounds [28,29]. The liberated NO is allowed to react with ozone to form electronically excited molecules of NO_2^* which then decays back to ground state with the emission of light in the near infra red region (600–3000 nm). The emitted light is allowed to pass through a filter (600–800 nm), the signal amplified, and detected using a 1-mV recorder. The TEA has been shown to be highly sensitive and specific to NO, and to give a linear response over a wide range of concentration. Since moisture interferes with the detection, the series of condenser and cold traps serve to remove it as much as possible.

2.2. Reagents

All reagents used were of analytical or high purity grade. Tetrabutylammonium hydrogen sulfate was purchased from Sigma, St Louis, MO, USA. All reagents were used without further purification. Water used for preparing reagents and mobile phase was distilled in an all glass apparatus. Sodium nitrite standard solutions were prepared fresh daily.

2.3. Samples

The cured meats and the baby food samples were purchased locally from retail outlets. Each sample was mixed well either by homogenizing in a blender (for the meats) or stirring with a spatula (for the baby foods) before taking an aliquot for analysis. The human saliva and the urine samples were made slightly alkaline (*ca.* pH 10), immediately after collection, by the addition of 2% NaOH solution, and then stored

at 4°C until analysis. This was done to prevent bacterial reduction of nitrate to nitrite, and to stabilize nitrite during storage.

2.4. Determination of nitrite

Before beginning analysis, the TEA and the post-column reactor were set up as described by Havery [27] with modifications as mentioned above. A series of duplicate injections (50 or 100 μl) of appropriate dilute nitrite standards (0.01 to 10 μg $NaNO_2$/ml) were made allowing approximately a 10-min gap between injections (the nitrite peak eluted within 6 min). The TEA attenuation was set between 16 and 1024 depending on the amount of $NaNO_2$ injected. A 4-min interval between injections was adequate in the flow injection mode for nitrite eluted within 1 min.

2.5. Standard curve for nitrite

Two sets of standard curves for sodium nitrite, one in the range of 0.5 to 100 ng per injection and the other between 0.1 to 1 μg per injection were constructed to determine the linearity of response over this entire range. Each standard was analyzed in duplicate, and an average of the peak heights were used to construct the curves. This was, however, not carried out regularly (done only once a month). For routine analysis the concentration of nitrite in a sample extract was calculated from its relative response to that of an appropriate standard giving a comparable (within ± 50%) response.

2.6. Analysis of cured meats and baby foods

A 10-g aliquot of a well homogenized sample was extracted with water, and the extract processed and filtered as described previously [30]. A 50–100 μl aliquot of the final filtrate was analyzed as above after appropriate dilutions (2 to 10-fold) with the HPLC mobile phase.

2.7. Analysis of urine and saliva

No sample preparation or clean-up was necessary for the analysis of these samples. They were analyzed as above after 10 to 100 fold dilutions with the HPLC mobile phase.

2.8. Colorimetric determination of nitrite

A method based on Griess colorimetric procedure [30] was used for the spectrophotometric determination of nitrite in cured meats and the baby food samples. It was also used for the analysis of human saliva samples, but because of the smaller size and lesser amounts of suspended solids in these samples, the method was operated on a micro scale. This involved mixing 2 ml sample with 1 ml of 2% NaOH solution, addition of 0.5 ml of 0.42 *M* $ZnSO_4$ solution (to precipitate proteins), heating the mixture for 15 min in a 50–60°C water bath, making up volume to 5 ml with water, centrifuging and pipetting out a 2-ml aliquot of clear supernatant, and finally determining the level of nitrite by the Griess colorimetric procedure as described previously [30]. The detection limit of the colorimetric method was about 1 ppm (μg/g) in each of the above substrates. Although no urine samples were analyzed by this method, this can be done, if desired, using the method for cured meats and baby foods [30].

3. Results and discussion

The chemiluminescence method described in this paper is based on an earlier procedure reported by Cox [22], but it differs significantly both in the design of the apparatus and in its operating conditions from those previously reported [22]. Cox used an apparatus in which nitrite was reduced to NO, by heating a 20-ml aliquot of nitrite standard or sample with various reducing agents, on a batch operation basis. After each analysis, the apparatus had to be dismantled, cleaned, and reassembled before resuming the next analysis. In our method a post-column reactor developed by Havery [27] for the determination of N-nitroso compounds was used. This allowed rapid successive determination of nitrite until the flask K became *ca.* 60% full. At that point, the flask could be emptied and analyses resumed, without dis-

mantling the apparatus, as described under Experimental. Furthermore, this technique has the added advantage that it can be used either in the flow injection or HPLC mode thus making the method versatile. The former mode is more suitable for the analysis of relatively dirtier samples which otherwise might reduce the life of an expensive HPLC column. In the HPLC mode, the retention time of the nitrite peak can be used as an additional parameter for its identification which makes the technique more specific. The same thing can be said about another chemiluminescence method [23] reported recently that is also operated on a batch operation basis, but uses a gas sampling valve that allows replicate analyses of the same sample in a single run.

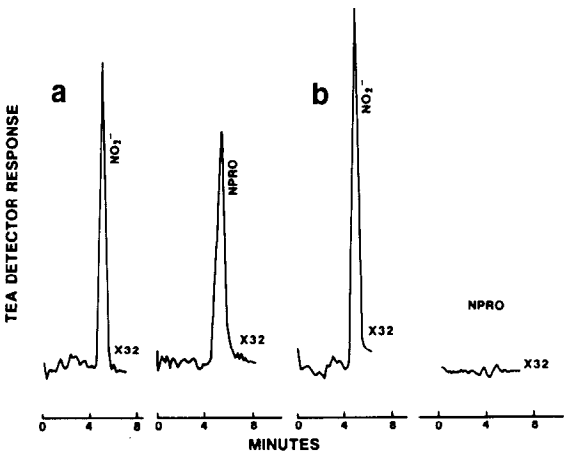

Fig. 2. HPLC chemiluminescence determination of nitrite. (a) With KI flowing into the reactor (2 ng $NaNO_2$ and 17.5 ng N-nitrosoproline (NPRO), respectively); (b) without KI and only glacial acetic acid flowing into the reactor (2 ng $NaNO_2$ and 17.5 ng NPRO, respectively). Mobile phase flow-rate, 1 ml/min.

4. Selectivity, sensitivity, and reproducibility

Since the method is based on the detection of NO, any compound producing NO under the post-column reactor conditions will give a false-positive response for nitrite. Previous research [25,27] has shown that most N-nitroso compounds and organic nitrites (*e.g.*, butyl nitrite) do give a positive response in such a system. Therefore, an attempt was made to minimize interferences from these compounds by varying the reagents in the reactor. It was observed that if the KI solution and the sulfuric acid–acetic acid mixture were replaced, respectively, with 10% ascorbic acid solution (in flask, F) and glacial acetic acid, the response from N-nitroso compounds became negligible. Omitting the ascorbic solution in flask F with only glacial acetic acid flowing into the reactor also gave no or negligible (*ca.* 1–5%) response for N-nitroso compounds (Fig. 2). In the absence of any reductant under the latter conditions, acid dismutation reactions, yielding NO from HNO_2, are responsible for the positive response from nitrite [23,24]. Since foods and biological materials contain only extremely low concentrations of N-nitroso compounds, their presence would not interfere with the determination of nitrite in these substrates provided the above modified conditions are used. Organic nitrites, however, may still give a positive response. It is highly unlikely, however, that these compounds would be present in foods in high enough concentrations to interfere with the determination of nitrite. Nitrate (as the Na salt) even at a relatively large amount (50 to 100 ng/injection) did not produce a detectable response.

In view of the above, most analyses were carried out without any KI solution flowing into

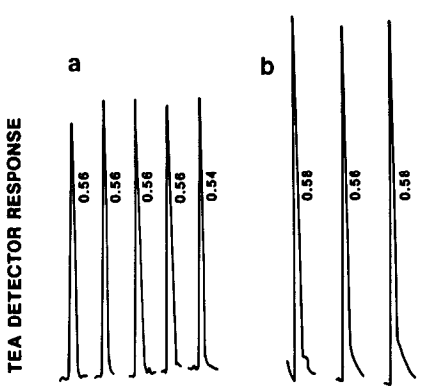

Fig. 3. Reproducibility of nitrite determination by flow injection chemiluminescence technique. (a) 5 replicate injections of 10 ng $NaNO_2$ each (attenuation, 256); (b) 3 replicate analyses of a cured turkey breast extract containing 26 ppm $NaNO_2$ (attenuation, 128). The peak elution times are printed beside each peak.

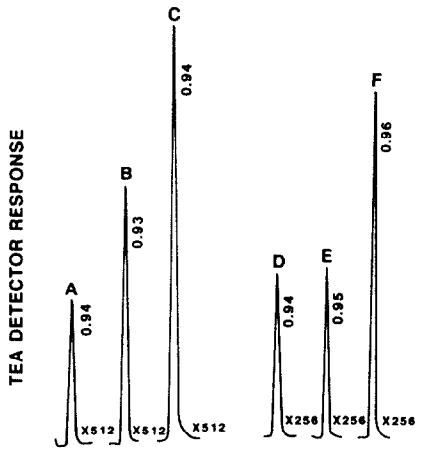

Fig. 4. Recovery studies using flow injection chemiluminescence determination. (A) 0.1 ml out of 400 ml final dilution of a pepperoni sample found to contain 11 ppm $NaNO_2$; (B) 0.1 ml of $NaNO_2$ spiking solution (spiking level, 20 ppm $NaNO_2$); (C) 0.1 ml out of 400 ml final dilution of the above pepperoni spiked with 20 ppm $NaNO_2$ (calculated recovery, 97.8%); (D) 0.1 ml of a human saliva sample after a 100-fold dilution; (E) 0.1 ml of $NaNO_2$ spiking solution (spiking level, 5 ppm $NaNO_2$); (F) 0.1 ml of above saliva after spiking with 5 ppm $NaNO_2$ and a 100-fold dilution (calculated recovery, 105%).

the reactor because this minimized the possibility of interference from N-nitroso compounds. The method worked highly satisfactorily both in the flow injection and HPLC modes, and was found to be highly sensitive and reproducible. The minimum detection limit ($>3\times$ noise level) seemed to be about 0.1 ng per injection. The response was linear over a wide range (1 to 1000 ng) of $NaNO_2$ injected. Replicate injections (n = 4 or 5) of both standards and sample extracts gave highly reproducible responses (Fig. 3), and these ranged between 0.2 to 4% for replicate injections of 50 ng (attenuation, 256) and 1 ng $NaNO_2$ (attenuation, 64; not shown in Fig. 3), respectively. Although distilled water was adequate as a mobile phase in the flow injection mode, for ease of operation, the HPLC mobile phase containing the ion-pair reagent was used throughout. In the HPLC mode, the nitrite ion usually eluted after 3 or 6 min depending on the mobile phase flow-rates (Figs. 2 and 5). With a more active (brand new) HPLC column, the retention time of nitrite was found to be slightly longer (not shown).

Table 1
Comparison of nitrite levels in some selected samples as determined by two different methods

Sample	Levels (ppm) of nitrite (as $NaNO_2$) detected	
	Chemiluminescence technique	Colorimetric method
Cured meats		
All beef wieners	17.1	18.3
All beef salami	33.5	30.7
Pepperoni	8.2	7.8
Human saliva		
Person A	3.7	3.1
Person B	8.2	6.5
Person C	3.8	4.4
Person D	13.1	10.6
Baby foods		
Junior mixed vegetables	0.11	Negative[a]
Beginner green beans	0.10	Negative
Beginner carrots	0.09	Negative
Strained vegetables and ham	0.15	Negative

[a] Detection limit, *ca.* 1 ppm.

5. Applications

The method was used to determine its suitability for the determination of nitrite in a few selected samples of foods and human biological materials. The average recoveries of sodium nitrite added to cured meats (spiking level, 10 ppm), human saliva (spiking level, 5 ppm), and human urine (spiking level, 0.02 ppm) were found to be 108% ($n = 3$), 105% ($n = 2$), and 92% ($n = 6$), respectively. A few representative chromatograms from these recovery studies are shown in Fig. 4. For comparison purposes, a few samples of cured meats, human saliva, and baby foods were reanalyzed by the above mentioned colorimetric procedure which is a well established method and has been shown to give highly reliable and accurate results in a number of laboratories [30,31–33]. The two sets of results compared well with each other except that the colorimetric method was not sensitive enough to detect the extremely low levels of nitrite in the baby foods, and hence gave negative results for these samples (Table 1). The overall detection limit of the chemiluminescence method was estimated to be about 5 ppb (μg/kg) which is 200 times lower than that of the colorimetric method. Moreover, the new method is much faster and requires very little sample preparation, especially in the flow injection mode. If needed, an analysis can be completed within 5 to 90 min depending on the sample analyzed.

Thus far, the majority of the experiments mentioned above were carried out using the flow injection mode. But, in a few cases, the sample extracts or the diluted samples (*e.g.*, saliva) were reanalyzed using the HPLC mode. Here again, the two sets of results agreed fairly well with each other (Fig. 5). Experiments along these lines should occasionally be carried out to double check the results obtained by flow injection analysis. This is particularly advisable for samples containing extremely low levels of nitrite or where the results appear to be abnormal. However, for samples containing high levels (> 10 ppm) of nitrite there is no need to use the HPLC column. The data obtained by using the flow injection technique should be reliable because the likelihood of interferences from N-nitroso compounds and organic nitrites at such high levels of nitrite is very remote. Also, for rapid monitoring purposes, where only an idea about the maximum possible level of nitrite present is required, the flow injection technique should be the method of choice.

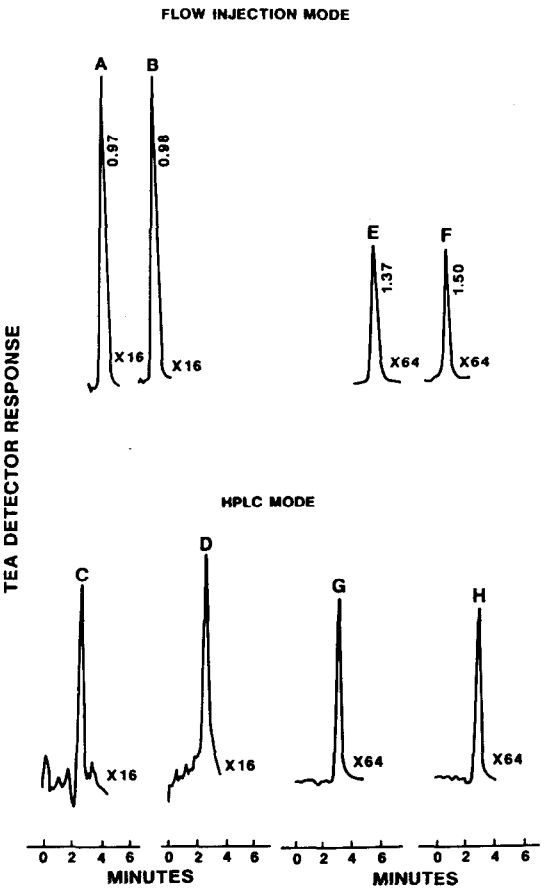

Fig. 5. Comparison of flow injection with HPLC chemiluminescence determination of nitrite. (A) 0.1 ml out of 400 ml final dilution of a baby food (carrot); (B) 0.5 ng $NaNO_2$ standard. Both A and B analyzed by the flow injection technique (sample was found to contain 0.17 ppm $NaNO_2$). (C) The same baby food extract and (D) the same standard analyzed by the HPLC technique (sample in this case was found to contain 0.18 ppm $NaNO_2$). (E) A human saliva sample and (F) 1.25 ng $NaNO_2$ standard both analyzed by the flow injection technique (sample was found to contain 1.3 ppm $NaNO_2$). (G) The same saliva sample and (H) the same standard as above both analyzed by the HPLC technique (sample in this case was found to contain 1.4 ppm $NaNO_2$).

In summary, we have developed a sensitive and rapid technique that is useful for both low and high level determination of nitrite in foods and biological materials. The technique should be particularly useful to the meat industry where critical control and monitoring of nitrite levels at various stages of manufacturing and storage of cured meat products are extremely important. Although TEA is a fairly expensive equipment, other commercially available chemiluminescence detectors, which are cheaper than a TEA, could be employed to carry out such analyses. It is hoped that other workers will find the technique useful.

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