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Rapid and Selective Electrochemical Determination of Nitrite in Cured Meat in the Presence of Ascorbic Acid

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Abstract. A simple, fast and selective amperometric procedure for nitrite determination in cured meat based on the use of a carbon paste electrode is presented. Direct interference of ascorbic acid, which interferes with electrochemical detection, was eliminated by applying a cellulose acetate membrane of 100 Da molecular weight cut-off on the electrode. Indirect interference of ascorbic acid due to its auto-oxidation reaction and its reaction with nitrite is discussed. In order to stabilise the ascorbic acid, metaphosphoric acid and EDTA were added.

In contrast to the standard spectrophotometric method, which requires careful control of pH and the use of carcinogenic reagents, amperometric determination of nitrite is simple, rapid, and works without reagents. Also, sample preparation is simpler because it requires neither heating to 80 °C nor the centrifugation steps of the official spectrophotometric method. The detection limit achieved was 14 ppb (0.2 μM) nitrite in the solution, which corresponds to a detection of 2.8 ppm in meat samples. The method was compared with the standard spectrophotometric procedure and applied to the detection of nitrites in cured meat samples with satisfactory results.

Key words: Nitrite determination; ascorbic acid interference; amperometric detection; carbon paste electrode.

Nitrite is added to cured meat in concentrations of less than 150 ppm in order to prevent the growth of microorganisms such as *Clostridium botulinum* that can cause food poisoning. In recent years, nitrite has been classified as a toxic compound because it can react with amines present in meat, leading to the formation of carcinogenic nitrosamines [1]. As a consequence, the level of nitrite in cured meat must be kept at a minimum level. For this reason a number of substitutes were tested and it became customary to add ascorbic acid in order to reduce the amount of nitrite added. Ascorbic acid is added in higher concentrations than nitrite because of its preservative and antioxidant properties.

Various methods for nitrite determination in meat have been proposed, the most popular being the AOAC spectrophotometric method which uses sulphanilamide and N-(1-naphthyl)ethylenediamine (NED) [2]. Unfortunately, recoveries of less than 50% were

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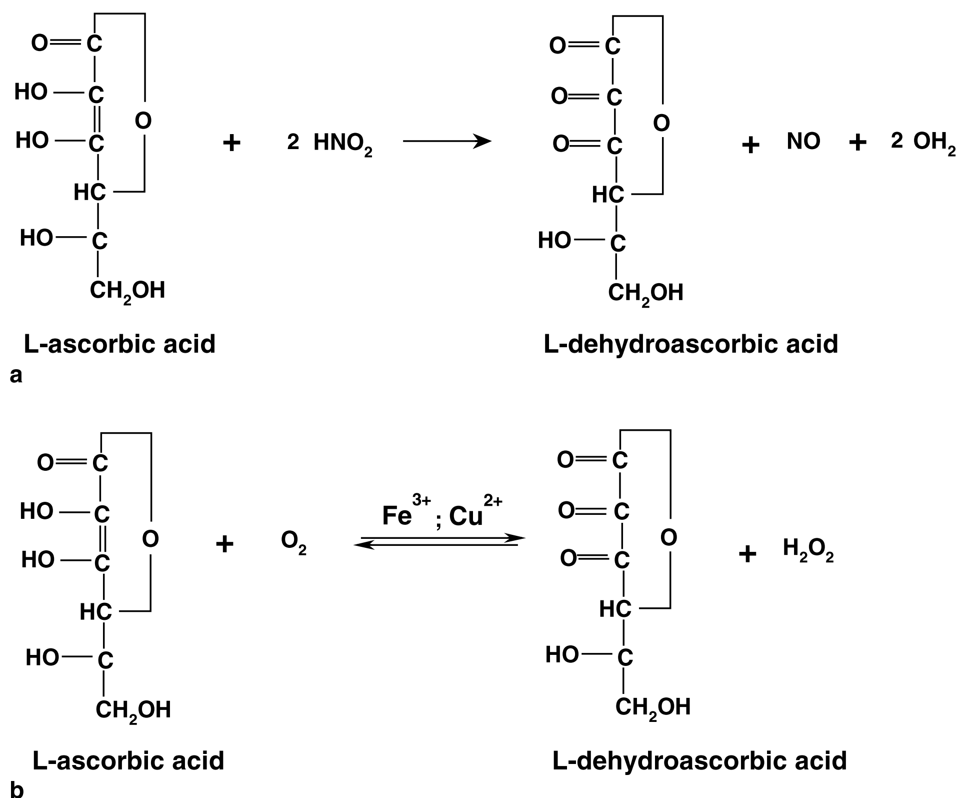

Fig. 1. Schemes for the reaction of ascorbic acid with nitrous acid (a) and auto-oxidation reaction of ascorbic acid (b)

reported for nitrite determination by this method because of the ascorbic acid reaction with nitrite [3]. The reaction between ascorbic acid and nitrite or nitrous acid was first observed by Karrer and Bendas [4]. It is a typical redox reaction, in which ascorbic acid reacts with nitrous acid (in acidic media of $pH < 5$), with the formation of dehydroascorbic acid and nitric oxide (Fig. 1a). Different methods have been used to reduce the ascorbic acid interference including the digestion of aqueous slurry of the sample at 80 °C for 2 h (AOAC Official Method) or the digestion of a slightly alkaline slurry at 80 °C for 1.5 h followed by clarification with zinc sulphate [5]. Binstok et al. [6] proposed an improved procedure of the Norwitz & Keliher method based on the use of sand during the digestion step and a larger quantity of NED during the diazotisation step. However, this method is time-consuming, requires careful control of acidity for the colorimetric step and uses carcinogenic reagents.

Alternative procedures based on nitrite reductase coupled with spectrophotometric detection [7], ion chromatography [8] or HPLC methods [9], were developed for nitrite determination in meat samples.

A number of electrochemical methods have been reported for nitrite determination [10–12], but only a few were applied to meat analysis. Ion selective electrodes (ISEs) offer attractive features for nitrite determination. Perez-Olmos et al. published an evaluation of the applicability of a nitrite-selective electrodes to different meat samples, but no studies of ascorbic acid interference were reported [13].

Nitrite can be oxidised at a potential of +0.9 V at glassy carbon (GC) [14, 15] and platinum electrodes [16] relative to the Ag/AgCl reference electrode. Unfortunately, the high potential required (+0.9 V) also causes direct oxidation of interfering species such as ascorbic acid. Ascorbic acid is an important electroactive interferent which is very easily oxidised at electrodes when a positive potential is applied. Size exclusion (e.g., *via* cellulose acetate, CA) [17], charge exclusion (e.g., *via* Nafion) [11] or size/charge exclusion (e.g., *via* poly(1,8 diaminonaphtalene)) [18] were applied in order to eliminate ascorbic acid interference. However, ascorbic acid can also interfere indirectly due to its oxidation reaction. In the presence of oxygen and in media of $pH < 5.5$, oxidation of

ascorbic acid leads to the formation of dehydroascorbic acid and hydrogen peroxide (Fig. 1b) [19].

It is therefore mandatory that any nitrite sensor for use in meat analysis must compensate for direct and indirect ascorbic acid interference.

We have already reported a selective method for nitrite determination in waters based on the elimination of direct interference by ascorbic acid using a Pt electrode covered with a cellulose acetate or various other electropolymerised membranes [18]. Here we propose a fast and selective amperometric procedure for nitrite determination in cured meat using a carbon paste electrode covered with a cellulose acetate membrane. This probe turned out to be free from interferences by ascorbic acid as present in meat samples.

Experimental

Reagents and Solutions

Sodium nitrite, sodium acetate, $ZnSO_4 \cdot 7H_2O$, sulphanilamide and N-(1-naphthyl)ethylene diamine · 2HCl (NED) were from Sigma Chemical Co, St. Louis, MO, (www.sigma-aldrich.com); L-ascorbic acid, acetic acid glacial, HCl 37%, monobasic and dibasic sodium phosphate and EDTA were from Carlo Erba, Milan, Italy, (www.carloerbareagenti.com). Metaphosphoric acid (pellets), graphite (1–2 μm) were from Aldrich, Milan, Italy (www.sigma-aldrich.com) and all other reagents of analytical grade were from Sigma.

Acetate buffer solution was prepared using 0.1 M sodium acetate, and its pH was adjusted to different pHs with acetic acid.

All solutions were prepared with bi-distilled water.

Apparatus

Amperometric measurements were carried out with an LC-4B amperometric detector (BAS, Bioanalytical System, Lafayette, USA, www.bioanalytical.com). Currents were recorded using home-made software created by Dr. S. Kalinowski (Olsztyn, Poland).

A potentiostat/galvanostat μ–AUTOLAB, type II (EcoChemie B.V., Ultrecht, The Netherlands, www.ecochemie.nl) was used for voltammetric studies. A Pt electrode (3 mm diameter, Model MF 2013 from BAS), a glassy carbon electrode (3 mm diameter) and a carbon paste electrode (3 mm in diameter) were used in all experiments. A combined auxiliary/reference electrode (Pt–Ag/AgCl/3 M KCl) (model 805/CPG/6 from AMEL, Milan, Italy) was used.

Preparation of Home-Made Cellulose Acetate Membranes

Cellulose acetate membranes were prepared dissolving 2 g of cellulose acetate (53%) and 20 mg of poly(vinyl acetate) (Carlo Erba, Milan, Italy, www.carloerbareagenti.com) in 30 mL acetone and 20 mL cyclohexanone [18]. A precision gauge tool (from Precision Gage and Tool Co., Dayton, OH, USA) was used to cast the cellulose membrane. Probes were assembled by placing the membrane on top of the electrode and securing it with an O-ring.

Electrochemical Procedure

The nitrite sensor polarised at +0.9 V *vs.* Ag/AgCl reference electrode was immersed in 10 mL of stirred acetate buffer solution and allowed to equilibrate. Aliquots of standard nitrite were then injected into the buffer and the current changes recorded.

Sample Treatment for Electrochemical Measurements

5 g of homogenized meat was mixed with 100 mL of cold (4 °C) 0.1 M acetate buffer (pH 4) containing 50 μM EDTA and 1% metaphosphoric acid. The mixture was vigorously stirred for 30 min at room temperature and then filtered through a 12.5 cm filter paper (medium texture; ashless).

0.5–1 mL of the filtrate were added into the electrochemical cell which contained 9–9.5 mL of an 0.1 M acetate buffer of pH 4 plus 50 μM EDTA and 1% metaphosphoric acid. The nitrite sensor was polarised at +0.9 V.

Spectrophotometric Procedure

For spectrophotometric determinations the Norwitz & Keliher method [5] was followed. 5 g of homogenised meat were added to approximately 70 mL 0.01 M NaOH and blended. This mixture was quantitatively transferred into a 300 mL Erlenmayer flask adding 0.01 M NaOH to reach a final volume of about 200 mL. The mixture was left to digest at 80 °C for 90 min (with occasional swirling). Then, 5 mL of 10% $ZnSO_4$ as clarifying agent was added to the hot solution. The resulting solution was stirred and cooled at room temperature, then diluted to 250 mL with bi-distilled water in a calibrated flask and filtered through a 12.5 cm filter paper (medium texture; ashless).

The sulphanilamide – NED reagent was prepared as follows: 0.5 g sulphanilamide was dissolved in 150 mL of 1 M hydrochloric acid at room temperature; 6 mL of 0.20% NED solution was added, and the solution diluted to 200 mL.

For colorimetric measurements 2–20 mL of filtrate was transferred to 25 mL calibrated flasks, 5 mL of sulphanilamide – NED reagent was added, and the final volume was made up to 25 mL with bi-distilled water. The absorbance was measured after 15 min, at 542 nm against a blank (obtained by mixing 2–20 mL of filtrate with 5 mL 1 M HCl, the final volume being 25 mL).

Results and Discussions

Following our previous work [18], the first amperometric experiments were performed with a Pt electrode assembled with a cellulose acetate (CA) membrane (cut-off 100 Da) and polarised to +0.9 V *vs.* Ag/AgCl using acetate buffer at pH 4. The permeability of this membrane to ascorbic acid was very low. Indeed, for a pH range between 4 and 8, no signal was registered at a relatively high concentration (1 mM) of ascorbic acid prepared from fresh 0.2 M stock solution. However, if a stock solution of low ascorbic acid concentration (<500 μM) is kept in contact with atmospheric oxygen at room temperature (by stirring), an oxidation process of ascorbic acid results in the generation of hydrogen peroxide.

The influence of the medium's pH on ascorbic acid decomposition is presented in Table 1. We chose to work with a concentration of ascorbic acid which may

Table 1. Influence of the medium on ascorbic acid decomposition

Medium	Signal (nA)
0.1 M acetate buffer, pH 4.0	2.60
HCl 10 mM, pH 2.0	0.10
NaOH 10 mM, pH 12.0	9.85
0.1 M phosphate buffer, pH 7.4	2.50

Protocol: 200 μM ascorbic acid stirred for 45 min in different media; 1 mL of this solution was added to 9 mL 0.1 M acetate buffer, pH 4 cellulose acetate/Pt electrode; E = +0.9 V.

be found in meat sample filtrates (according to our experimental protocol). For this study, 200 μM ascorbic acid solutions prepared at different pH values were stirred for 45 min before recording the electrochemical response after 10-fold dilution (final concentration 20 μM).

It can be clearly observed that a significant signal appeared when working with a slightly acidic or neutral pH. This signal could be attributed only to the hydrogen peroxide generated by the oxidation of ascorbic acid. At alkaline pH, a very high signal is observed which is not attributable only to hydrogen peroxide, but rather to various products caused by the decomposition of ascorbic acid at alkaline pH, as reported by Yuan and Chen [20]. They examined the degradation of ascorbic acid in aqueous solutions in the range of pH 1–10 and also reported that the concentration of the degradation products of ascorbic acid was very low around at pH 4.0.

Oxidation of ascorbic acid can be catalysed by trace amounts of Cu^{2+} or Fe^{3+} [19, 21] or by light [22] (Fig. 1b). Zambonin and Losito [23] reported that hydrogen peroxide even forms at pH 7, but in lower amounts than the expected mole ratio of 1:1. This hydrogen peroxide can give positive signals when an amperometric electrode modified with CA, nafion or a polymer is used for the analysis of food samples or biological compounds in complex matrices.

In the clinical field, the interference of ascorbic acid is reduced by the use of enzymes to remove it, or by cations to promote auto-oxidation, or different ascorbic acid quenchers to eliminate interference with fluorescence assays [24, 25].

For ascorbic acid stabilisation, different agents are reported, the one most commonly used being metaphosphoric acid [26, 27]. Also, EDTA is frequently used for the complexation of ferric and cupric ions. We studied the influence on the electrochemical response of ascorbic acid stirred for 45 min in different buffers which contained 50 μM EDTA and 1% metaphosphoric acid (Table 2).

200 μM ascorbic acid solutions prepared in different buffers were stirred for 45 min before recording the electrochemical response after 10-fold dilution (the final concentration in the electrochemical cell was 20 μM). It was demonstrated that at $pH < 5.5$ and in the presence of EDTA and metaphosphoric acid, auto-oxidation of ascorbic acid with hydrogen peroxide generation does not occur. The small signal obtained at pH 8.0 was probably due to hydrogen peroxide produced by the uncatalysed auto-oxidation of ascorbic acid [19] and/or due to some electroactive molecules produced by the degradation of ascorbic acid at alkaline pH [20]. In further experiments, EDTA and metaphosphoric acid were added to the working buffer (acetate buffer, pH 4.0) as stabilizing agents for ascorbic acid.

These simple experiments proved that the auto-oxidation of ascorbic acid which takes place in acetate buffer with hydrogen peroxide generation can be stabilized using EDTA and metaphosphoric acid.

Since hydrogen peroxide, under particular conditions, is a product of auto-oxidation of ascorbic acid and this gives a large signal at the Pt electrode, we studied other electrode materials such as glassy carbon (GC) and carbon paste (CP) which are less sensitive to hydrogen peroxide and more sensitive to nitrite. Thus, we compared the electrochemical response of standard solutions of hydrogen peroxide and nitrite at the surface of Pt, CP and GC electrodes using linear sweep voltammetry (Fig. 2). For both

Table 2. Influence of pH on ascorbic acid stabilisation

Medium	Signal (nA)
0.1 M phosphate buffer, pH 8.0 with 50 μM EDTA and 1% metaphosphoric acid	1.40
0.1 M acetate buffer, pH 5.5 with 50 μM EDTA and 1% metaphosphoric acid	no signal
0.1 M acetate buffer, pH 5.0 with 50 μM EDTA and 1% metaphosphoric acid	no signal
0.1 M acetate buffer, pH 4.0 with 50 μM EDTA and 1% metaphosphoric acid	no signal

Protocol: 200 μM ascorbic acid stirred for 45 min in different media; 1 mL of this solution was added to 9 mL 0.1 M acetate buffer, pH 4.0 cellulose acetate/Pt electrode; E = +0.9 V.

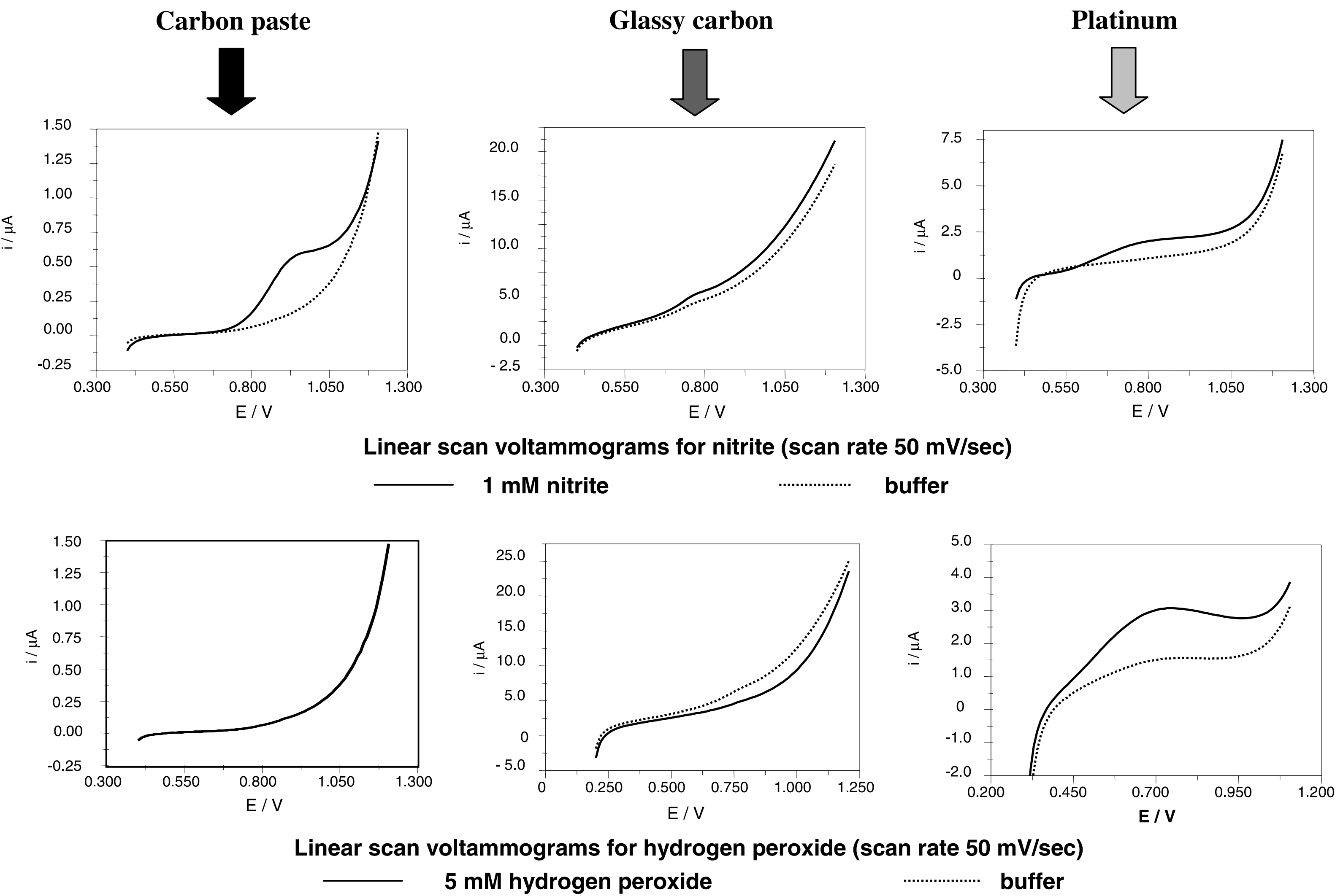

Fig. 2. Influence of the electrode materials on the electrochemical oxidation of nitrite and hydrogen peroxide

carbon paste and Pt electrodes, well shaped peaks for nitrite oxidation around +0.9 V were recorded, while for GC only a small peak was recorded. In the case of hydrogen peroxide electro-oxidation, no clear peaks were recorded in the range of the potential studied using CP and GC electrodes. For the Pt electrode, as expected, an oxidation peak for hydrogen peroxide with a maximum at +0.65 V was observed.

In conclusion, we decided that CP was the best electrode material to use for nitrite determination in the presence of hydrogen peroxide. Also, in amperometric conditions, the CP electrode assembled with a CA membrane is 10 times more sensitive for nitrite determination than a Pt electrode assembled with the same membrane (results not shown). Further studies were thus carried out using the CP electrode assembled with the CA membrane.

To eliminate direct interference of ascorbic acid in nitrite determination, the CP electrode was assembled with a CA membrane. Figure 3 shows the calibration curve for nitrite determination using the CP electrode with and without the CA membrane. In both cases, good linearity was observed over the concentration range of the nitrite studied, but sensitivity turned out to be lower for the electrode assembled with the membrane, because of the low diffusion rate of the nitrite through the CA membrane. The equations were: $I = -12.74 + 111.25 \cdot c$; $r^2 = 0.9991$ for naked CP electrode and $I = -0.66 + 2.54 \cdot c$; $r^2 = 0.9998$ for the CP electrode with CA membrane, where I was the current in nA; c was the nitrite concentration in μM and r^2 was the correlation coefficient. The detection limit (S/N = 3) was 0.01 μM nitrite for a naked CP electrode and 0.2 μM for the CP electrode assembled with CA membrane.

For nitrite determination using the CP electrode with CA membrane, an extended linear range up to 0.5 mM and an RSD of 1.82% (n = 10; sodium nitrite concentration = 5 μM) were obtained. Under our experimental conditions (1 mL of filtrate added to 9 mL of working buffer in the electrochemical cell and using a CP electrode assembled with CA membrane), the calculated detection limit of sodium nitrite in meat was 2.8 ppm. Usually, concentrations between 10–150 ppm nitrite are found in meat products.

Figure 4 shows the amperometric response of the CP electrode assembled with CA membrane to nitrite, hydrogen peroxide and ascorbic acid. The results clearly demonstrate the selectivity of the proposed sensor towards nitrites.

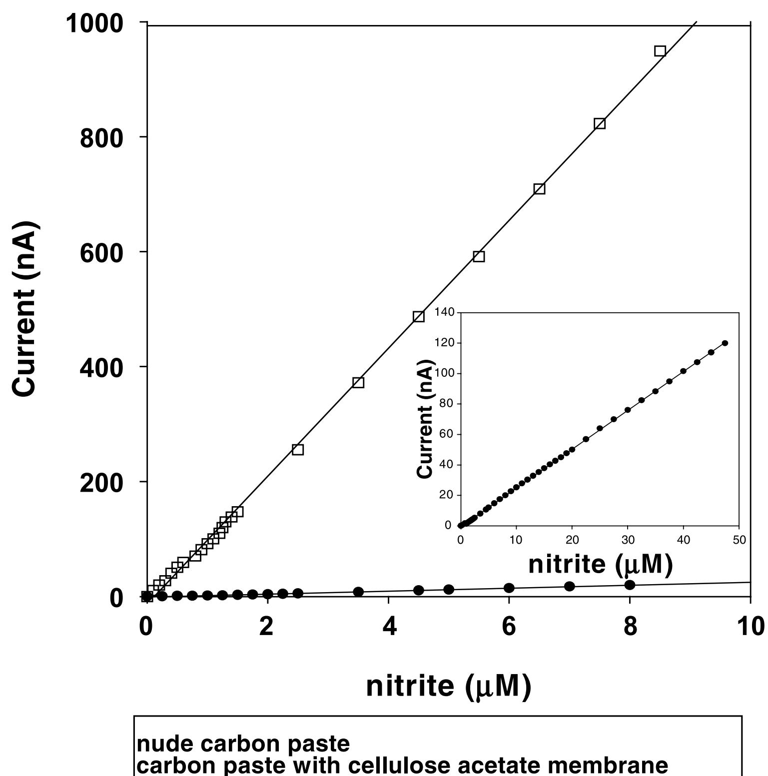

Fig. 3. Calibration graph for nitrite determination using a carbon paste electrode with and without cellulose acetate membrane (insert: extended calibration graph for carbon paste electrode assembled with cellulose acetate membrane). Working buffer: 0.1 M acetate buffer, pH 4.0 containing 50 μM EDTA and 1% metaphosphoric acid; E = +0.9 V *vs* Ag/AgCl

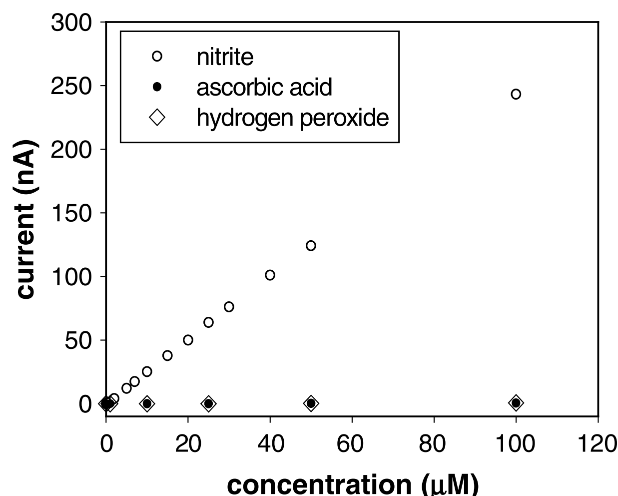

Fig. 4. Amperometric response for nitrite, ascorbic acid and hydrogen peroxide using a CP electrode assembled with CA membrane. Working buffer: 0.1 M acetate buffer, pH 4.0 containing 50 μM EDTA and 1% metaphosphoric acid; E = +0.9 V *vs* Ag/AgCl

The response of the CP electrode assembled with CA membrane to a high concentration of fresh ascorbic acid solution (1 mM) was also checked, and no signal was recorded, indicating that ascorbic acid does not cross the membrane.

Real Sample Analysis

The AOAC official method for nitrite determination in meat is based on the Griess method which uses N-(1-naphthyl)ethylenediamine (NED) and sulphanilamide (SA). To reduce ascorbic acid interference, the AOAC method provides a heating step of 2 h at 80 °C of the aqueous slurry of the sample. Yet this type of sample treatment is not very efficient and also requires a centrifugation step. Although the AOAC method is very sensitive, low recoveries are reported when used for nitrite determination in meat due to the ascorbic acid interference [3]. Norwitz and Keliher [5] have proposed modifying the AOAC method (referred to here as the modified AOAC method) using an alkaline medium (0.01 M NaOH) for the digestion step and introducing a new step based on addition of a clarifying agent ($ZnSO_4$) for protein precipitation. In this way the centrifugation step can be replaced with a simple filtration step. However, we observed that this method requires careful control of the pH in all steps. We thought that the low recoveries observed in the modified AOAC method were due only to the pH effect. Indeed, the pH of the filtrate can change the final pH of the Griess reaction, which will result in a lower recovery. After sample treatment with sodium hydroxide, the pH of the filtrate was in some cases alkaline, which resulted in a high final pH in the Griess reaction. Thus, we proposed to adjust the pH of the filtrate to 6.5 ± 0.5. Table 3 reports the comparison between spectrophotometric methods based on AOAC and modified AOAC methods with the pH of filtrate adjusted to 6.5. It can be seen that the reproducibility is improved and the absorbance of the filtrate (before adding the reagents NED and sulphanilamide) is decreased. We also found that ascorbic acid interfered with nitrite determination in meat samples using the AOAC method when the ratio of ascorbic acid/nitrite (w/w) was higher than 25. This is the case when a low amount of nitrite (<10 ppm) is present together with a high amount of ascorbic acid (>250 ppm) in meat.

A number of cured meat samples (Italian specialities purchased from local markets in Rome) were analysed for nitrite concentration using the further modified AOAC method and the newly developed electrochemical method. In order to evaluate the recovery of the electrochemical method, these samples were also analysed with and without spiking the meat sample with 25 ppm nitrite (Table 4). The results obtained with the electrochemical method were in good agreement with those obtained with the spectrophotometric method. It can also be noted that good recoveries are obtained even at low concentrations of nitrite (<10 ppm).

Table 3. Comparison between AOAC and modified AOAC spectrophotometric methods for nitrite determination in meat

Analytical parameter	N (number of samples)	AOAC method	Modified AOAC method*
RSD (relative standard deviation)	5	4.1%	1.3%
Absorbance of the filtrate	5	0.030–0.060	0.005–0.010
Recovery for 20 ppm nitrite	3	86%, 102%, 105%	99%, 102%, 89%
Recovery for 100 ppm nitrite	3	93%, 98%, 94%	99%, 99%, 91%
Interference of ascorbic acid (AA)		yes if $AA/NO_2 > 25$	no

* The pH of the filtrate was adjusted to 6.5 ± 0.5.

Table 4. Nitrite determination in real samples

Sample	Electrochemical method		Colorimetric method nitrite found (ppm) (B)	Relative error (%) (A–B)/B · 100
	Nitrite found (ppm) (A)	Recovery (for 25 ppm nitrite)		
Salami (*Type Milano*)	8.3	96%	8.6	−3.6
Cooked ham I (*Prosciutto cotto*)	6.5	108%	4.9	+32.6
Cooked ham II (*Prosciutto cotto*)	ND	95%	ND	–
Parma ham (*Prosciutto crudo*)	ND	99%	ND	–
Wurstel I (*Suillo classico*)	113.3	80%	109.9	+3.1
Bologna sausage (*Mortadella*)	ND	98%	ND	–
Wurstel II (*Suillo*)	58.2	81%	55.7	+4.5

ND Not detectable.

Conclusions

In this paper we have proposed a new sensor for the detection of nitrites in meat, and we have also studied the direct and indirect interference of ascorbic acid. The problem of determining nitrite in meat samples was solved by applying a CA as a permselective membrane to the CP electrode and using acetate buffer of pH 4.0 containing EDTA and metaphosphoric acid as stabilizing solution of ascorbic acid in the sample preparation phase. The amperometric method of determination carried out in this way was shown to be very simple, reagentless, and reproducibile and also required less time for sample preparation. Moreover, its application to nitrite analysis in cured meat samples produced satisfactory results.

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