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Application of flow injection analysis to determine protein-bound nitrite in meat products

C. Ruiz-Capillas *, P. Aller-Guiote, F. Jimenez-Colmenero

Department of Meat and Fish Science and Technology, Instituto del Frío, (CSIC), C/Jose Antonio Novais, 10, Ciudad Universitaria, 28040 Madrid, Spain

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Abstract

The development and application of a methodology based on flow injection analysis (FIA) for the determination of protein-bound nitrite (PBN) in meat products was studied. Since the FIA methodology used for measuring residual nitrite was not appropriate for determining PBN (even at a concentration of 15.9 mg of PBN/kg) in meat products, the procedure was modified and then studied for residual nitrites and PBN. Ammonium chloride (A), which is used conventionally (the original FIA method), was replaced by different carriers (the modified FIA method): B (buffer 7); C (buffer 7.5); D (buffer 8); E (NaOH 0.5 M) and F (NaOH 1 M). Carriers B and C provided the lowest limits of quantification of residual nitrite, lower than that obtained using the original FIA method. The method for determining PBN in several meat products (frankfurter and dry sausages) was validated by comparing it with the method usually used. The results obtained indicate that the modified FIA method (with carriers B and C) can be used as a simple, easy, fast, accurate and precise methodology for quantifying residual nitrite and PBN in meat products.

Keywords: Meat products; Protein bound nitrite; Flow injection analysis; $NaNO_2$

1. Introduction

It is known that nitrites added to meat products become rapidly depleted as a result of such factors as heat treatment, product composition, storage temperature, etc. Residual nitrite levels in foodstuffs are extremely important, partly because of their potential to react with amines and amides to form carcinogens, and partly because of their contribution as a source of nitrite in human nutrition (Lee, Cassens, & Fennema, 1976; Pegg & Shahidi, 2000). Recently, interest in nitrite and its reaction has re-emerged because of its possible negative effect (epidemiological suggestions that hot dog consumption is linked to childhood cancer) and positive effect (protective roles of nitric oxide) on human health (Cassens, 1997). In the last decades, the meat industry has modified the technologies for cured meat production resulting in lower levels of residual nitrite (Cassens, 1997; EFSA, 2003).

Numerous studies have been done on the different reactions between nitrite and various naturally occurring chemical components in biologically complex systems such as meat, not only to understand the curing mechanism but also to assess the potential health hazards (Cassens, Greaser, Ito, & Lee, 1979; Cassens, Ito, Lee, & Buege, 1978). Different facts show that a substantial amount of the nitrite added to meat for curing is bound to or reacts with muscle proteins (Cassens et al., 1979). Protein-bound nitrite (PBN) in meat systems has been studied by several authors (Carballo, Cavestany, & Jiménez-Colmenero, 1991; Dennis, Massey, & Mcweeny, 1980; Ito, Cassens, Greaser, Lee, & Izumi, 1983; Moiseev, Cornforth, & Egbert, 1995; Olsman & van Leeuween, 1977).

The most common procedure for determining PBN has been the one used by Mirna (1970) and modified by Olsman and van Leeuween (1977). This method is based on the ability of Hg^{2+} to release the NO-group from nitrosothiols and other forms of bound nitrite, and then the nitrite content is measured colorimetrically with the Griess

* Corresponding author. Tel.: +34 915445607; fax: +34 915493627.
E-mail address: claudia@if.csic.es (C. Ruiz-Capillas).

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reagent (Olsman & van Leeuween, 1977). Several methods have been reported for quantitative determination of residual nitrite in meat products also based on colorimetric determination of the Griess reaction, which has been adopted as an AOAC official method of analysis (AOAC, 1990; Butt, Riaz, & Iqbaz, 2001; Liang, Iwatsuki, & Fukasawa, 1994; Ötzekin, Nutku, & Erim, 2002). However, most of these methods are tedious to execute and are not very economical because they use large volumes of toxic reagents, are time-consuming and involve intense handling (Andrade, Viana, Guadagnin, Reyes, & Rath, 2003). In contrast, other authors have developed a much more accurate, fast and precise method by flow injection analysis (FIA), also based on the Griess reaction. This method offers the advantage of being simple with a high analytical sampling rate and uses low-cost equipment (Gine, Bergamin, Zagatto, & Reis, 1980; Higuchi & Motomizu, 1999; Pinho, Ferreira, Oliveira, & Ferreira, 1998).

This paper describes the development and application of a methodology based on flow injection analysis for determining protein-bound nitrite in meat products.

2. Materials and methods

2.1. Samples

Different meat products were used for the experiment: cooked products (frankfurter sausages) and Spanish dry sausages (''salchichón'' and ''chorizo'') obtained from the local market. The products were from different manufacture lots. At least 100 g of each of the samples were homogenised and used in the determinations indicated below.

2.2. Reagents

All the chemicals used were of analytical-reagent grade obtained from Sigma-Aldrich and Fluka (Sigma-Aldrich Company Ltd, GmbH), and from Panreac (Spain). Distilled, deionised water was used throughout this research.

The flow injection analysis reagents were prepared as follows:

- *First reagent* (carrier) was prepared with ammonium chloride (25 ± 0.0001 g) and 0.5 ± 0.0001 g of EDTA (ethylenediaminetetraacetic), which was dissolved in 1000 ml of miliQ water and its pH was adjusted to 8.5 with concentrated ammonia.
- *Second reagent* (colour reagent): Sulphanilamide (20 ± 0.0001 g) and *N*-(1-Naphthyl)-ethylendiamino-dihydrochloid monomethanolat (NED) (1 ± 0.0001 g) plus 100 ml of ortophosphoric acid was dissolved in 1000 ml of miliQ water.
- Standard nitrite solutions with concentrations of 0.125–4 mg of $NaNO_2$/l were prepared from $NaNO_2$, a stock solution of 1000 mg of NO_2/l.
- Phosphate buffers (with pH 7, 7.5 and 8) and sodium hydroxide (0.5 M and 1 M) were also prepared.

2.3. Procedures

2.3.1. Determination of protein-bound nitrite (PBN)

PBN was determined according to Olsman and van Leeuween (1977). With this method it is possible to obtain a ''PBN extract'' which in the form of free nitrite contains protein-bound nitrite. The nitrite content in the ''PBN extract'' was determined colorimetrically with the Griess reagent. The results were expressed as mg of $NaNO_2$/kg of the sample.

2.3.2. Determination of residual nitrite by flow injection analysis (the original FIA method)

The determination of $NaNO_2$ using the FIA technique was performed according to the methodology used by Gine et al. (1980) and Pinho et al. (1998) with modifications. The extract used for determinations of $NaNO_2$ was prepared from 10 g of the sample according to (AOAC, 1990, method 973.31). This extract of samples was injected into the Flow Injection Analysis manifold through a 100-μl loop which was then closed. The first reagent (ammonium chloride) was driven by the peristaltic pump at a flow rate of 1 ml/min to the reodine valve where the sample or standards were collected and then carried to a first reaction coil. Then the mixture continued until it came to a ''T'' where the colour reagent was added, also driven by the peristaltic pump at a flow rate of 1 ml/min. All this passed to a reaction coil of 100 cm where the colour reaction occurred. This colour reaction produced a response in the detector that was connected to a chart recorder where the response was collected in the form of a peak. The response was proportional to the concentration of nitrite in the sample or in the standard solutions. The peaks produced on the chart recorder by known concentrations of standard nitrite solutions were used to calibrate the scale. The determination of nitrite was based on the reaction with sulphanilamide to form a diazonium salt which was added to NED to form azo dye compounds whose absorbance was measured spectrophotometrically at 540 nm.

2.3.3. Improved flow injection analysis (the modified FIA method) for determination of PBN

In order to adjust FIA to determine PBN, different reagents were assayed replacing the one used in the original FIA method (ammonium chloride, carrier A). These reagents were: B (buffer 7); C (buffer 7.5); D (buffer 8); E (NaOH 0.5 M) and F (NaOH 1 M).

2.3.4. pH

The pH was determined on a Radiometer model PHM 93 pH-meter (Meterlab, Copenhagen, Denmark) at room temperature on homogenates of meat products in water in a ratio 1:10 (w/v).

2.4. Statistical analysis

Regression analysis was used to adjust the standard straight lines with Excel. Mean values were compared with

an F test ($P < 0.05$) in the Statgraphic 2.1 programme (STSC, Rockville, Md., USA).

3. Results and discussion

As mentioned earlier, the determination of PBN by the Olsman and van Leeuwen method (1977) is based on the ability of Hg^{2+} to release the NO-group from nitrosothiols and other forms of bound nitrite, and then the nitrite content (in the PBN extract) is measured colorimetrically with the Griess reagent. This kind of colorimetric determination of nitrite that has been described has some disadvantages, which naturally also affect the determination of PBN. These limitations could be resolved by measuring the nitrite content in the "PBN extract" by flow injection analysis in a similar way to how residual nitrite is determined. Thus the PBN was analysed (from the "PBN extract") in the same conditions assayed for determining residual nitrite. The results obtained from the analysis of PBN in several meat products (Table 1) showed that this methodology was not appropriate for quantifying PBN since it was unable to detect the presence of PBN concentrations (even of 15.9 mg/kg product) which were found within the measurement range of the method for determining residual nitrite by flow injection analysis.

The fact that it was not possible to quantify PBN by the original FIA method could be related to the different pH in each of the extracts used for its measurement: "residual nitrite extract" (pH 8.3–8.6) and "PBN extract" (pH 6.1–6.3). This means that the colorimetric reaction, which is conditioned by the pH (Usher & Telling, 1975), does not take place in both instances in similar pH conditions (results not shown). The optimum pH of the PBN extract for PBN determination was established within the range 5.0–6.5 (Olsman & van Leeuween, 1977), therefore the pH of the "PBN extract" obtained in this experiment was within the range expected. To prepare the reaction medium, different carrier reagents were assayed instead of the ammonium chloride used in the original FIA method (Table 2). The pH range selected for the different carrier reagents was between those usually assayed for determining $NaNO_2$ by flow injection analysis (Gine et al., 1980; Pinho et al., 1998; Higuchi & Motomizu, 1999; Andrade et al., 2003).

Using standard nitrite solution, standard straight lines were obtained in the different assay conditions (Table 2). The results obtained indicated that the quantification limit (QL) of improved flow injection analysis (the modified FIA method) was, except for one case, lower than the original FIA method. The lower quantification limit (QL) was obtained using carriers B and C (buffers 7 and 7.5).

Cooked and dry sausages were used to determine residual nitrite using both procedures: the original FIA method and the modified FIA method (Fig. 1). In frankfurters the amount of residual nitrite determined by the original FIA method was 70.62 mg/kg, whereas in the modified FIA method it ranged from 67.48 to 73.77 mg/kg. The greatest concentration of residual nitrite ($p < 0.05$) was obtained using carrier B (buffer pH 7). Residual nitrite levels of dry sausages ("chorizo" and "salchichón") could not be quantified since their concentration was lower than the QL of the original FIA method (Table 2). Conversely, using the modified FIA method (with carrier reagents B and C) it was possible to quantify the residual nitrite in both products: residual nitrite was 2–3 mg/kg for "chorizo" and 7–8 mg/kg for "salchichón" (Fig. 1). Although it was highlighted that concentrations <10 mg/kg product were assimilated to zero residual nitrite (EFSA, 2003), a survey on residual nitrite levels in processed meat referred to quantities of residual nitrite well below this concentration in numerous meat products (Rincón León, Zurera Cosano, Polo Villar, & Lora, 1983; Cassens, 1997; EFSA,

Table 1
Determination of protein-bound nitrite (PBN, mg of $NaNO_2$/kg) in frankfurters and dry sausages ("salchichón" and "chorizo") by the Olsman and van Leeuwen method (1977) and original FIA method

Sample	Olsman and van Leeuwen (1977)	Original FIA method
Frankfurter sausage	15.90 ± 0.02	Not detected
Chorizo	0.73 ± 0.03	Not detected
Salchichón	0.19 ± 0.02	Not detected

"Chorizo" and "salchichón": Spanish ripened-dry fermented sausage products.

Table 2
Regression curve and regression coefficient (R) for determination of $NaNO_2$ by original FIA method using carrier A (ammonium chloride) and modified FIA method using different carriers B (buffer 7); C (buffer 7.5); D (buffer 8); E (NaOH 0.5 M) and F (NaOH 1M)

FIA	Carriers	Regression ($y = ax + b$)			QL (mg $NaNO_2$/l)
		a	b	R^2	
Original method	A	2.904	−0.437	0.998	0.185
Modified FIA method	B	3.023	−0.081	0.998	0.054
	C	3.112	−0.091	0.997	0.049
	D	3.123	−0.178	0.995	0.095
	E	3.590	−0.198	0.997	0.080
	F	3.630	−0.528	0.997	0.270

QL quantification limit; calculated as the sum of concentration corresponding to ten times the SD of the background noise, obtained with 10 determinations. y: peak height; x: $NaNO_2$ mg/l.

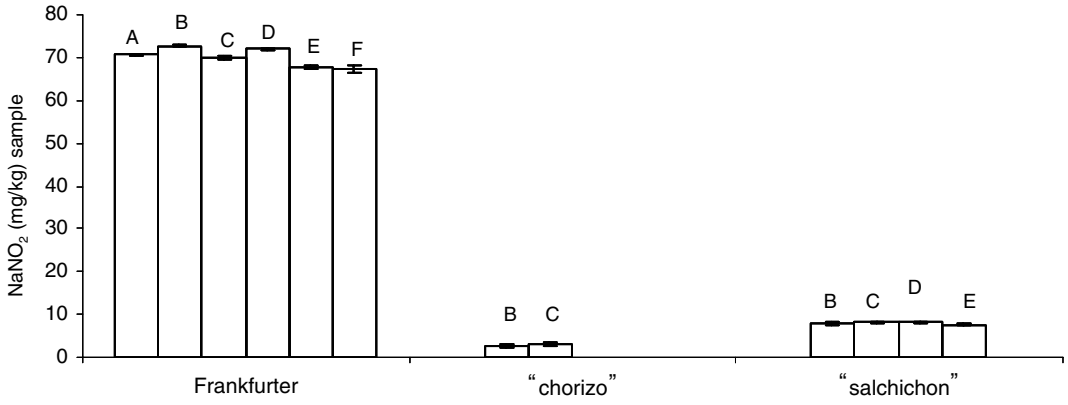

Fig. 1. Determination of residual nitrite (mg/kg sample) in frankfurters and dry sausages (''salchichón'' and ''chorizo'') by flow injection analysis: original FIA method using carrier reagent A (ammonium chloride) and modified FIA method using different reagents B (buffer 7); C (buffer 7.5); D (buffer 8); E (NaOH 0.5 M) and F (NaOH 1 M).

2003; Bozkurt & Erkmen, 2004). Thus, for example, levels of 0.40–78.97, 0.51–62.55, and 1.53–103.06 mg/kg were found in dry sausages (''salchichón'' and ''chorizo'') and frankfurters, respectively (Rincón León et al., 1983). These results reveal that the modified FIA method has considerable advantages for determining residual nitrite compared with the procedure initially used, the original FIA method.

In order to test the validity of the modified FIA method for determining PBN in meat products, PBN was quantified in cooked and dry sausages by applying the Olsman and van Leeuween (1977) procedure as the reference method and compared with the modified FIA method (Fig. 2). Generally, the results exhibited similar trends to those observed in determining residual nitrite using the modified FIA method (Fig. 1). In the case of frankfurters, similar PBN levels ($p > 0.05$) were detected when the Olsman and van Leeuween (1977) procedure and the modified FIA method were applied using the carrier reagents B and C. This same trend was observed in the PBN of dry sausages: ''salchichon'' and ''chorizo'', which, however, could not be determined with the other assayed carrier reagents (D, E, and F) (Fig. 2). In frankfurters the highest PBN levels ($p < 0.05$) were obtained using the carrier reagent D (buffer pH 8) (Fig. 2). Yet when the carrier reagents E and F (NaOH 0.5 and 1 M) were assayed, the PBN levels detected were lower ($P < 0.05$) than those determined with the Olsman and van Leeuwen technique (1977) (Fig. 2). The different PBN values in Fig. 2 and Table 1 for the same kind of products could be the result of using different manufacture lots, as described in materials and methods.

The highest PBN levels were detected in frankfurter sausages with levels of up to 12 mg of $NaNO_2$//kg. Lower levels (almost half) of PBN were detected in the dry sausages ''salchichón'' and ''chorizo''. The lowest levels were detected in ''salchichón'', less than 4 mg of $NaNO_2$/kg. Generally, the PBN levels detected in the different products studied (Fig. 2) were similar to those obtained by other authors in similar products (Olsman & van Leeuween, 1977; Carballo et al., 1991), and their presence was condi-

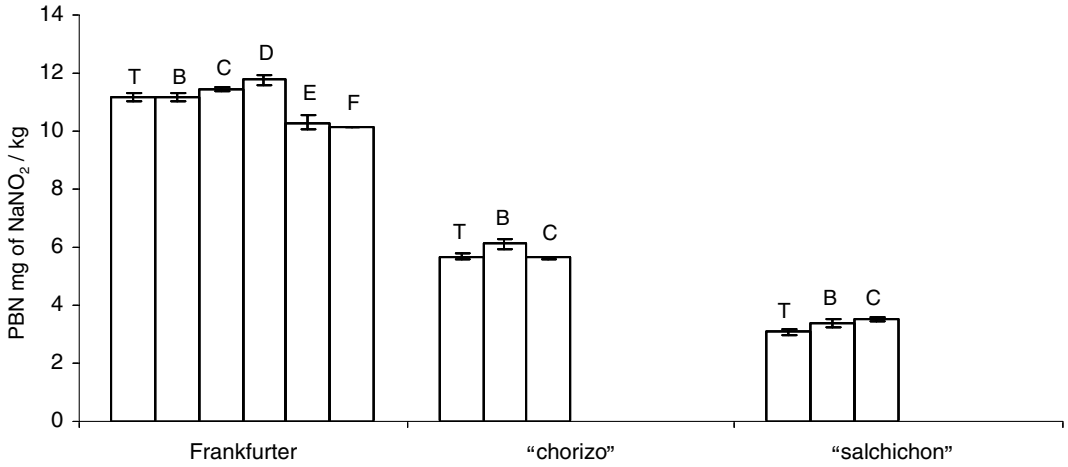

Fig. 2. Determination of protein-bound nitrite (PBN) (mg of $NaNO_2$/kg sample) in frankfurters and dry sausages (''salchichón'' and ''chorizo'') by the Olsman and van Leeuwen procedure (1977) (T) and modified FIA method using reagents B (buffer 7), C (buffer 7.5), D (buffer 8), E (NaOH 0.5 M) and F (NaOH 1 M).

tioned by different factors like processing conditions, temperature, storage, light, etc. (Carballo et al., 1991).

Analysis of the PBN results obtained when the Olsman and van Leeuwen procedure (1977) and the modified FIA method (Fig. 2) were compared indicate that the modified FIA method (buffers pH 7 and 7.5) can be an alternative to the Olsman and van Leeuwen method for evaluating protein-bound nitrite. Thus it is possible to exploit the advantages of flow injection analysis for evaluating PNB, among others, its greater sensitivity, also apparent in the determination of other compounds, assay speed (allows a higher number of samples to be determined in the same period of time) and it is also time saving. Moreover, this method is much cleaner, since it works in a continuous system where reagents are not handled all the time (Ruiz-Capillas & Horner, 1999).

4. Conclusions

For the determination of residual nitrite and PBN in meat products by flow injection analysis it was found that the buffer phosphates pH 7.0 and 7.5 (the modified FIA method) were more appropriate as carrier reagents than ammonium chloride which is used for determining nitrite in the original FIA method. The results obtained suggest that the modified FIA method would be a suitable alternative to the Olsman and van Leeuween (1977) method for the determination of PBN.

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