

Validation of spectrophotometric method to detect and quantify nitrite in ham pate

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The objective of this work was to validate the spectrophotometric method to detect and quantify nitrite using ham pate as a source. The validated analytical conditions were 540 nm wavelength and the samples reading between 40 to 70 minutes after addition of coloring agents. The tested performance criteria were: linearity, matrix effect, selectivity, detection and quantification limit, accuracy, precision and robustness. As the results have obtained linearity in the studied zone (0.125 to 3 g/mL) and matrix effect was not observed, there was not any interference from ascorbic acid, but interference occurred from sodium eritorbate, in concentrations above 2.5 mg/100g. The detection limit was defined as 12.5 mg of nitrite/kg for pate and the quantification limit as 25 mg of nitrite/kg of pate. The method presented repeatability and reproducibility. The accuracy was assessed through recovery values varying from 84 to 110%. Respecting robustness, it was observed that the variations applied to the extraction procedure depended on the concentration of nitrite in the pate sample. In conclusion, the methodology was validated under the tested conditions.

Uniterms: Validation. Nitrite. Spectrophotometric method. Ham pate.

O objetivo deste trabalho foi validar o método espectrofotométrico para determinação de nitrito usando como matriz patê de presunto. As condições analíticas usadas foram comprimento de onda de 540 nm e leitura das amostras entre 40 e 70 minutos após a adição dos reagentes complexantes. As características de desempenho testadas foram linearidade, efeito de matriz, seletividade, limite de detecção do equipamento e do método, limite de quantificação, exatidão, precisão e robustez. A linearidade foi obtida na faixa de trabalho estudada (0,125 a 3 µg de nitrito/mL) e não foi observado efeito de matriz. A concentração de ácido ascórbico na amostra não interferiu na análise e o eritorbato de sódio possuiu interferência negativa para concentrações acima de 2,5 mg/100g de amostra. O limite de detecção foi definido em 12,5 mg de nitrito/kg de patê e o limite de quantificação em 25 mg de nitrito/kg de amostra. O método apresentou repetitividade e reprodutibilidade intra-laboratorial. A exatidão foi avaliada através dos valores de recuperação que variaram entre 84 e 110%. Na avaliação da robustez, as variações realizadas no procedimento de extração foram dependentes do nível de concentração existente na amostra. Concluiu-se que a metodologia pôde ser validada nas condições testadas.

Unitermos: Validação. Nitrito. Espectrofotometria. Patê de presunto.

INTRODUCTION

The Ministry of Agriculture, *Livestock* and Sup-

plying (MAPA) regulates the use of nitrate and nitrite in meat products, and according articles 365, 372, 373 from RIISPOA, Brazil (1952), a maximum of 200mg of nitrite/kg of product is tolerated in meat products and, separately, a maximum of 1 part per thousand of nitrate. When sodium and potassium nitrates and nitrites are used in any combination, the amount of nitrite cannot surpass 200mg/kg. Nitrates and nitrites can be used as sodium

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nitrite (NaNO_2), potassium nitrite (KNO_2), sodium nitrate (NaNO_3), potassium nitrate (KNO_3), and its combinations. When nitrate is used, it is required the use of some reducer bacteria to transform it into nitrite, however this change is slow and difficult to control. Therefore, the direct addition of a well-known amount of nitrite makes the cure process easier and diminishes the color and taste variation in cured products (Chichester, Tanner Jr., 1972).

Nitrates and nitrites are additives largely used in meat industry, and they prevent the growth of *Clostridium botulinum*, provide the products with a pinkish color, cured meat typical flavor, and possess antioxidant effects (Kilic, 2000). However, these additives can cause serious damages to health; the nitrite combines with the secondary and tertiary amines present in the meat, creating nitrosamines, that are highly carcinogenic substances. They can also combine with hemoglobin creating methemoglobin, which damages the blood oxygen transport. The long term nitrite intake can cause methemoglobinemia, mainly in children, and the nitrite intoxication causes vasodilatation, smooth muscle relaxation, face redness, gastrointestinal discomfort, headache, cyanosis and vomiting. The n-nitrate compounds formed from the nitrite reactions with the amines are mutagenic and teratogenic (Harada, Silva, 2002).

Several techniques are used to detect and quantify nitrites in meat products. Between them, liquid chromatography, gas chromatography and UV/visible spectrophotometry. Between the advantages of the chromatographic methods, are not only the simultaneous detection of nitrites and nitrates, but also their fast analysis; however, it is an analytical process with high cost equipments and reagents (Schuster, Lee, 1987). The spectrophotometric analyses have been largely used for the detection and quantification of nitrate and nitrite in meat products, vegetables, legumes and water. Their advantages are the low cost method, and the fact that they meet the analyte accomplishment characteristics (Pringues *et al.*, 1995).

The validation of an analytical method is made by the assessment of its performance criteria. The most common performance criteria are: selectiveness, which is the method capacity to identify the specific investigated compound between many others; linearity, which is the method capacity to obtain results that are directly proportional to the analyte amount that is present in the sample; precision, which is the the proximity obtained in the result, respecting to many measurements developed with the same sample; accuracy, which is the proximity of obtained results to the real analyte amount into the sample; detection limit, which is the minimum amount of analyte that is possible to detect; quantifying limit, which is the minimal amount of analyte present in the

sample that can be quantified; and robustness, which is the rehearsal method capacity in resisting from certain variations in the analytical parameters (Leite, 1996; Eucharem, 1998; Brasil, 2003).

The purpose of this study was to validate spectrophotometry, as nitrite detection and quantifying method in ham pate.

MATERIAL AND METHODS

This experiment took place in the Physical/Chemical Sector of Animal Source Products of the National Agricultural Laboratory (LANAGRO), in Pedro Leopoldo – MG, according Brazil methodology (1990). A Genesys 10UV sample reader spectrophotometer was used. The analytical conditions used were as follows: 540 nm wavelength and optical time to read the samples after the complexating reagents addition, varying from 40 to 70 minutes.

The evaluated performance criteria were linearity, matrix effect, selectiveness, detection and quantification limits, precision, accuracy and robustness. For the performance criteria, the following concentrations of the evaluation standard-solution of sodium nitrite were used: 0.125; 0.250; 0.5; 1.2; and 3 $\mu\text{g/mL}$, and ham pate blank matrixes were added at 12.5; 25; 50; 100; 200 and 300 concentrations of nitrite/product kg. The concentration of 200 mg of nitrite/product kg, the maximum value allowed by law, corresponds to the 2 $\mu\text{g/mL}$ standard-curve. The standard-curve was repeated in all days of analysis.

RESULTS AND DISCUSSION

Linearity

The determination coefficients (r^2) evaluated in the linearity vary from 0.9943 to 0.9976, being all of them superior than the usually required ones, proposed by Chasin *et al.* (1998), who has recommended coefficients above 0.98.

Selectiveness

Ascorbic acid test

The results about the selectiveness test using ascorbic acid as an interfering are shown at Table II.

It is observed at Table I that the concentration values obtained in the sample analyses containing nitrite and ascorbic acid, were not different from the values of samples containing only nitrite. This result shows that ascorbic acid has not interfered over nitrite spectrophotometric analyses, at any studied concentration.

TABLE I – Material and methods used at each performance criterion tested

Performance criterion	Analysis conditions and zone	Number of repetitions	Statistical method	Reference
Linearity	0.125; 0.250; 0.5; 1; 2 and 3 µg/mL	All the analyses days	Linear regression Residue graph	
Matrix effect	0.125; 0.250; 0.5; 1; 2 and 3 µg/mL without matrix 12.5; 25; 50; 100; 200 and 300 mg/kg with matrix	3	T-test 95% of Confidence Interval	Sampaio (2002)
Selectiveness	Nitrite = 200 mg/kg 3.25; 6.5; 13 mg ascorbic acid /pate 100 g Nitrite = 200 mg/kg 2.5; 5; 7.5 mg sodium erythorbate/pate 100 g	3 5	Variance analysis Random delimitation Confidence Interval of 95%	Sampaio (2002)
Equipment detection limit	Ham pate blank samples	10	“0 + 3s” s = sample standard- curve	Eucharem (1998) Brasil (2003) Inmetro (2003)
Method detection limit and quantification limit	12.5; 25; 50; 100; 200 and 300 mg/kg	6	Acceptable precision and accuracy values	(Community, 2002)
Accuracy	12.5; 25; 50; 100; 200 and 300 mg/kg	6	Recuperation value Acceptable variation gap	(Community, 2002)
Precision	Repeatability 12.5; 25; 50; 100; 200 and 300 mg/kg	6	Horwitz equation Comparison of found CV with 10.7% CV value	(Community, 2002)
	Reproducibility 25; 200; 300mg of sodium nitrite / kg of pate Sample weight 5 and 10 g Extraction method: vortex 2 min and boiling water bath 15 min	3	Horwitz equation Comparison of found CV with 16% CV value Tukey test Trustiness degree 95%	Sampaio (2002)
Robustness				

Sodium erythorbate test

The results about the selectiveness test using sodium erythorbate as an interfering are shown at Table III.

As observed, the sodium erythorbate has interfered in the nitrite spectrophotometric analyses over a sodium erythorbate concentration of 2.5 mg/100 g of pate. The nitrite concentration average obtained in the samples containing nitrite plus 2.5 mg of sodium erythorbate /100 g of pate had the same statistical value as the control group (containing only nitrite). However, the nitrite sample averages containing nitrite plus 5 mg of sodium erythorbate/100 g and nitrite plus 7.5 mg of sodium erythorbate/100 g were statistically different from the control group.

TABLE II – Ascorbic acid addition effect in nitrite quantification. All pate samples have the concentration of 200mg/kg of ascorbic acid

Ascorbic acid addition (mg/100 g)	nitrite (mg/kg)
0	204.53 ^a ± 13.05
3.25	173.07 ^a ± 28.03
6.5	197.32 ^a ± 13.91
13	193.58 ^a ± 13.89

Non-capitalized letters in the column indicates different statistical averages by Tukey test (P<0.05).

TABLE III – Sodium erythorbate addition effect in nitrite quantification. All pate samples have the concentration of 200mg/kg of sodium erythorbate

Sodium erythorbate addition (mg/100g)	nitrite (mg/kg)
0	209.32 ^a ± 1.9
2.5	189.08 ^{ab} ± 5.3
5	173.05 ^b ± 22.1
7.5	134.05 ^c ± 20.2

Non-capitalized letters in the column indicates different statistical averages by Tukey test (P<0.05).

Matrix Effect

The matrix effect was evaluated through the angle coefficient (a) value comparison obtained in samples with and without matrix. The angle coefficient values were 0.9136; 0.9525 and 0.9535 for the standard curves with matrix standard addition, and 0.9118; 0.9543 and 0.9648 for the pattern curves without matrix standard addition. The angle coefficient average values were compared by the t-test, in which the t calculated (0.0982) was inferior than the tabulated t (4.032) being the confidence interval of 95%, so the matrix effect was not identified (Sampaio, 2002).

Detection and quantification limit

For the definition of equipment detection limit, some tests using the “0+3s” formula have been made (Inmetro, 2003). The blank matrix obtained average values of 7.225mg of nitrite/kg of product and the standard deviation “s” was 0.78, therefore the value obtained by the utilization formula was 9.56mg of nitrite/kg of pate.

For the determination method of detection limit, a recovery average of twelve nitrite samples added at the concentration of 12.5mg/kg was observed, and the detection

limit method was defined as 12.5mg of nitrite/kg of pate. This result was based on acceptable precision and recovery values according to Community (2002), in which the acceptable recovery values vary from 80 to 110%, and the acceptable variation coefficient is of 10.7% for repeatability and 16% intra-laboratory reproducibility conditions (Community, 2002). The recovery values obtained in this concentration varied from 35 to 84%, but only one recovery value, in twelve samples, above 80% was found. For the precision values, in both analyzed levels, the nitrite concentration of 12.5mg/kg of pate was the only one that obtained variation coefficient values higher than the values accepted by the Directiva 2002/657/CE (Community, 2002).

The quantification limit was of 25 mg of nitrite/product kg and this was the second minor concentration used in the standard curve. Twelve blank matrix nitrite analyses added were done at this concentration, and the recovery average of these samples was of 81% and the variation coefficient was of 10.3%, reaching the acceptable precision and accuracy criteria according Directiva 2002/657/CE (Community, 2002).

Precision

Repeatability

According to Directiva 2002/657/CE from Community (2002), the acceptable variation gap for the precision in repeatability conditions is of 10.7%. This value was calculated starting from the Horwitz equation ($CV = 2^{(1-0.5 \log c)}$) used for intra-laboratory reproducibility. In the repeatability case the CV value should correspond to 2/3 of the calculated value for intra-laboratory reproducibility.

The results found in the analyses repeatability are demonstrated at Table IV. The variation coefficient among six independent repetitions at the same level of nitrite concentration was within the established standards of the Directiva 2002/657/CE from Community (2002).

TABLE IV – Concentration values obtained in repeatability from six repetitions of six analyzed nitrite concentrations, and respective variation coefficient values

Concentration (mg/kg)	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6	CV (%)
25	23.36	21.51	21.73	*	20.99	22.38	4.2
50	50.70	50.96	51.06	48.90	49.81	51.73	2.0
100	105.48	110.42	107.21	107.17	110.66	105.35	2.1
200	197.41	201.99	206.93	207.32	206.41	205.65	1.9
300	*	295.56	293.29	293.18	292.77	294.58	0.4

* Missing numbers during the procedures.

TABLE V – Obtained concentration values from intra-laboratory reproducibility, after six repetitions, from six analyzed nitrite concentrations and respective variation coefficient values

Concentration (mg/kg)	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6	CV (%)
25	18.77	18.67	16.88	17.40	21.09	19.93	8.3
50	51.42	51.10	50.36	49.63	51.84	51.63	1.7
100	106.39	103.02	106.28	107.33	105.54	106.07	1.4
200	210.64	206.85	202.32	208.01	207.80	208.22	1.3
300	291.20	293.2	290.89	295.52	290.15	293.73	0.7

Reproducibility

At Table V it is possible to observe the obtained results from the analyses done by other analyst, but using the same laboratory and equipment. The concentration levels studied (25, 50, 100, 200 and 300 mg/kg), in six totally independent repetitions, obtained the variation coefficient within the standards established by Directiva 2002/657/CE from the Community (2002), which is 16% as demonstrated in the Horwitz equation. In the equation $CV = 2^{(1-0,5 \log c)}$, c is the used mass fraction and, in this case, $c = 10^{-6}$, because the used mass fraction was mg/kg. Table V shows the obtained variation coefficient from the analyses done by the analyst in the intra-laboratory reproducibility.

To calculate the variation coefficient values obtained for the reproducibility, Tables IV and V were considered, representing the obtained values in the analyses made by the two participant analysts of this performance characteristic. The obtained variation coefficient values for the intra-laboratory reproducibility were as following (Table VI).

TABLE VI – Standard deviation method and obtained variation coefficient values for the intra-laboratory reproducibility

Concentration (mg/kg)	Pattern deviation	Variation coefficient (%)
25	2.087	10.3
50	0.921	1.8
100	2.116	2.0
200	3.571	1.7
300	1.785	0.6

In all studied concentration, the obtained variation coefficient values for the intra-laboratory reproducibility were inferior to 16%, according to the Horwitz Equation (Community, 2002).

Accuracy

At Table VII, the obtained recovery values in the accuracy test are presented.

TABLE VII - Obtained recovery values in the accuracy, respective acceptable variation gaps in the studied concentration zone, and variation coefficients

Concentration (mg/kg)	Recovery values (%)	Acceptable variation gaps (%)	CV values (%)
25	84 to 93	80 to 110	4.1
50	97.8 to 103.5	80 to 110	2.0
100	105.4 to 110.7	80 to 110	2.2
200	98.7 to 103.7	80 to 110	1.9
300	97 to 98	80 to 110	0.4

In the nitrite concentrations of 25; 50; 100; 200 and 300 mg/kg of pate, the found recovery values were within the acceptable variation gap. It was observed that as higher the analyzed concentrations, as higher the recovery values; and as lower the observed variation coefficient (thanks to higher amount of analyte), as lower the interference caused by losses. It is possible to observe that the recuperation values are within the acceptable gap, according to Directiva 2002/6.57/CE, in all studied concentrations (Community, 2002).

Robustness

The amount of weighed sample for the spectrophotometric nitrite analyze is dependent on the nitrite concentration present in the sample, according the analyzed results obtained (Table VIII). However, for the sample containing 25 mg of nitrite/kg of pate, it is recommended to weigh 10 g of pate, according to the traditional methodology (Brasil, 1999). For the samples containing 200 or 300 mg of nitrite/kg of pate, 5 g of sample should be weighed for the nitrite spectrophotometric analyze, which facilitates the analyte extraction process, thanks to the easier procedure of weighing 5 g instead of 10 g, when a large amount of analyzes are made in a single day, considering that for

the nitrite analysis, the analyte extraction should be made in the same day.

TABLE VIII – Nitrite concentration analyzed in robustness weighing 5 and 10g of sample, and obtained results in statistical analysis

Concentration (mg/kg)	Sample mass	
	5g	10g
25	16.51 ^a	22.96 ^a
200	213.86 ^a	204.90 ^b
300	310.03 ^a	287.05 ^b

Non-capitalized letters in the column indicate different statistical averages by Tukey test ($P < 0.05$).

As it could be observed at (Table IX), the extraction method used in the analytical procedure was dependant on the nitrite concentration contained in the sample. In the sample containing 25 mg of nitrite/kg of pate, both extraction analyte methods were efficient. However, for the concentrations of 200 and 300 mg of nitrite/kg of pate, the recommended analyte extraction is of 15 minutes in a boiling water bath, because it was statistically superior, when compared to results obtained in the analyte extraction for two minutes in vortex. To create a pattern, it is recommended the analyte extraction by bath of boiling water for 15 minutes, because this method covers efficiently all studied concentrations.

TABLE IX – Nitrite concentrations analyzed for robustness, using both proposed methods, and obtaining results in statistical analyses

Concentration (mg/kg)	Extraction methods	
	Vortex	Boiling water bath
25	19.62 ^a	19.86 ^a
200	211.58 ^b	207.18 ^a
300	297.45 ^b	299.62 ^a

Non-capitalized letters in the column indicate different statistical averages by the Tukey test ($P < 0.05$).

CONCLUSION

According to all tested conditions in this study, the spectrophotometric analytical methodology for nitrite detection and quantification in ham pate was validated.

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