Norihide Ando and Yukiharu Nagata

Laboratory of Chemistry and Technology of Animal Products
Kyushu University, Fukuoka, Japan

A Method for the Determination of Nitrite in Meat Products.

Because of the employment of various food additives in
processing meat products, the mechanism of the formation of
cured meat color has become extremely intricate. Not only
to clarify this but also from the standpoint of food hygi-
ene, it is of profound importance to determine the accurate
amount of nitrite in meat products.

It has recently been shown in our laboratory than in an
aqueous model system the accurate amount of nitrite cannot
be estimated by any of the colorimetric methods usually
employed for the determination of nitrite in foodstuffs
when nitrite coexists with any reducing substances, and
that this is due to the fact that nitrite decomposes ra-
pidly at the low pH range required for the formation of
azo dye by diazotization and coupling in the presence of
reducing substances 1,2).

Using an Orange I reagent as the color-developing reagent,
therefore, a lot of studies have been made and a successful
method which can estimate the accurate amount of nitrite
coexisting with various food additives in an aqueous model
system has been developed 2).

In the previous work 3), it has also been found that when
nitrite coexists with reducing substances in an aqueous

model system, the decomposition rate of nitrite increases with decreasing pH and increasing temperature. Since the decomposition of nitrite takes place even at the natural pH range of meat products, special attention should be given ' to the condition of extracting nitrite from meat products.

Several methods have been proposed for the determination of nitrite in meat products 4, 5, 6), but in these methods no elaborate investigations seem to have been made either on the condition of extracting nitrite from meat products or on the interference by various food additives usually employed in meat processing and reducing substances naturally existing in meat products.

The purpose of the present work is to find out the condition under which nitrite can be extracted completely from meat products without decomposition and to device a colorimetric method by which the accurate amount of nitrite in meat products can be estimated.

Experimental Work and Discussion

1. Effects of pH and heat treatment on the decomposition of nitrite coexisting with reducing substances in an aqueous model system.

   No particular study seems to have ever been made on the appropriate condition under which nitrite is to be extracted from meat products. But, as nitrite decomposes at the natural pH range of meat products, i.e., at the pH range about from 5,5 to 6,5, in the presence of reducing substances, special care must be taken to prevent the decomposition of nitrite during the process of extracting.

   In the AOAC method 4), which is widely employed for the determination of nitrite in meat products, nitrite is extracted with distilled water from finely comminuted sample by heating at about $80^{\circ}C$ on a steam bath for 2 hours with occasional shaking. In this case, as nitrite is extracted

from meat products, at the pH range about from 5,5 to 6,5 in the presence of such reducing substances as sulfhydryl groups of muscle proteins, other naturally existing substances and the remaining ascorbate or/and isoascorbate added, it is possible that the decomposition of nitrite may take place during the process of extraction.

To confirm this and to find out the extracting condition under which nitrite can be extracted completely from meat products with-out decomposition, the effects of pH and heat treatment on the decomposition of nitrite coexisting with reducing substances were investigated to begin with, using an aqueous model system.

(1) Preparation of sample solutions
Each sample solution was prepared by dissolving 0,002 % $NaNO_2$, 0,02 % sodium ascorbate and 0,02 % cysteine in each of the veronal buffer solutions of pH 5, 6, 7, 8, 9 and 10, respectively.

In the experiment with an aqueous model system, cysteine was used for observing the effect of sulfhydryl groups of muscle proteins.

Into a glass tube with a well-fitting glass stopper 20 ml of each sample solution was measured and heated at $80^{o}C$ for 60, 90 and 120 minutes respectively, then the remaining amount of nitrite in each sample solution after heat treatment was determined.

(2) Determination of nitrite in sample solutions
The amounts of nitrite in sample solutions were determined by a colorimetric method proposed by the authors in the previous paper 2), using Orange I reagent.

As a result of experiments with aqueous model system, it has been found that among the food additives usually employed in meat processing ascorbate,

isoascorbate and hexametaphosphate, besides cysteine, interfere with this colorimetric method.

In the proposed method, therefore, potassium ferricyanide is added first to an aqueous model system containing nitrite and various food additives to oxidize the reducing substances. Potassium ferrocyanide formed by the reaction of potassium ferricyanide with reducing substances interferes with this colorimetric method too, lead acetate, consequently, is added to precipitate it, whereupon hexametaphosphate is also precipitated by lead acetate simultaneously. By filtering off these precipitates, the filtrate becomes absolutely free from interfering substances, and accordingly the accurate amount of nitrite coexisting with various food additives can be estimated by determining the nitrite in the filtrate colorimetrically following the procedure given below.

(i) Orange I reagent: warm 360 ml of distilled water and 50 ml of glacial acetic acid to $50^{\circ}C$ and pour into a dark glass reagent bottle (600 ml) containing 0,25 g of powdered sulfanilic acid. Shake until dissolved, then add 0,2 g of -naphthol and dissolve by shaking. Cool the solution with running tap water to room temperature, and adjust the pH of which to 4,0 $\pm$ 0,05 with 10 % aqueous ammonia solution.

(ii) Potassium ferricyanide solution: Dissolve potassium ferricyanide in distilled water to give a final concentration of 10 mM.

(iii) Lead acetate solution: To prevent the formation of white turbidity by CO_2, dissolve lead acetate in CO_2 free distilled water, adjust the pH of which with 10 % acetic acid solution to

5,5, dilute with CO_2 free distilled water to
give a final concentration of 0,1 M, then filter.

(iv) Procedure: Immediately after the heat treatment
at $30^{\circ}C$, cool 20 ml of each sample solution to
room temperature with running tap water, adjust
the pH of which to 7,0 with 0,1 N HCl or 0,1
N NaOH, cool to $0^{\circ}C$ with ice water, add 10 mM
$K_3Fe(CN)_6$ solution until the color of the sample
solution gives a faint yellow, keep at $0^{\circ}C$ for
30 minutes in ice water, add 10 ml of lead
acetate solution, let stand at room temperature
for 10 minutes, dilute with distilled water to
200 ml, filter, to 5 ml. of the filtrate add
5 ml of Orange I reagent, keep at $30^{\circ}C$ for 30
minutes, cool with running tap water for 10 mi-
nutes, then measure the absorbance at 479 mμ.

(3) Effects of pH and heat treatment on the decomposition
of nitrite
The results given in Fig. 1 indicated that the de-
composition rate of nitrite increased with decrea-
sing pH and increasing heating time at $80^{\circ}C$ in the
pH range below 8,0, principally in the pH range
from 5,0 to 6,0, but that no decomposition took
place in the pH range above 8,0, i.e., from 8,0 to
10,0, even after being heated for 120 minutes at
$80^{\circ}C$ in the presence of reducing substances.

2. Condition of extracting nitrite from meat products

(1) Effect of pH on the amount of nitrite extracted
from cooked sausage

As it has come to be clear in an aqueous model
system that the decomposition rate of nitrite varies
with pH, expecially in the pH range from 5,0 to 6,0,
in the presence of reducing substances by heating
at $80^{\circ}C$, the effect of pH on the amount of nitrite

extracted at $80^{\circ}C$ from cooked sausage was investigated.

(i) Preparation of cooked sausage: From the beef chuck at three days after slaughter connective tissue and fat were removed as far as possible, then the beef was ground three times with a grinder. Immediately after mixing thoroughly with 2 % NaCl, 0,02 % $NaNO_2$ and 0,1 % sodium ascorbate, the ground beef was stuffed into a Krehalon (vinylidene chloride-vinyl chloride co-polymer) casing and cooked at $75^{\circ}C$ for one hour, then stored in a refrigerator at $4^{\circ}C$ until use.

(ii) Extraction of nitrite from cooked sausage: Two grams of cooked sausage sample was homogenized with 10 ml of distilled water for 3 minutes in a Waring blender and then diluted to 150 ml with distilled water. To each 150 ml of meat slurry of cooked sausage prepared in this way, 5 ml of 0,5 M socium diethylbarbiturate aqueous solution was added, and the pH of which was adjusted with 1 N HCl or 1 N NaOH to 6,0, 7.0, 8.0, 9.0 and 10.0 respectively. They were transferred into a 200 ml volumetric flask each, heated at $80^{\circ}C$ in a water bath for 90 minutes with occasional shaking, then the amount of nitrite extracted was determined by the procedure given below.

(iii) Determination of nitrite extracted: Immediately after the extracting-treatment at $80^{\circ}C$ for 90 minutes, cool each of the meat slurry samples at various pH values with running tap water for 10 minutes, adjust the pH of which to 8.0 with 1 N HCl or 1 N NaOH, cool in ice water for 30 minutes, add 3 ml of 10 mM

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$K_3Fe(CN)_6$ solution, keep at $0^\circ C$ for 30 minutes in ice water, add 10 ml of 0.1 M lead acetate solution, let stand at room temperature for 10 minutes, dilute with distilled water to 200 ml, filter, to 5 ml of the filtrate add 5 ml of Orange I reagent, keep at $30^\circ C$ for 30 minutes, cool with running tap water for 10 minutes, then measure the absorbance at 479 mμ.

In this procedure besides the interfering substances, proteins existing in the meat slurry of cooked sausage are precipitated by the addition of lead acetate, and by filtration a quite clear filtrate absolutely freed from interfering substances is obtained. The accurate amount of nitrite extracted, therefore, can be estimated colorimetrically by determining the nitrite in the filtrate following the procedure given above.

(iv) Effect of pH on the amount of nitrite extracted: The results given in Fig. 2 indicated that the amount of nitrite extracted increased with increasing pH in the pH range from 6.0 to 7.5. At pH 7.5 it reached a maximum and maintained almost the same value until pH 10.0.

Attention must be paid to the fact that the amount of nitrite extracted decreases markedly with decreasing pH in the pH range from 6.0 to 7.0, because this is the natural pH range of meat products and in the AOAC method nitrite is generally extracted at this pH range.

) Adjustment the pH of meat slurry to 9.0
In the present work it has become clear that nitrite does not decompose at all in the pH range from 8.0 to 10.0 by heating at $80^\circ C$ for 120 minutes even in the presence of reducing substances, so that the pH value

of 9.0 has been chosen as a recommendable pH value
at which nitrite is to be extracted from meat pro-
ducts.
To each 150 ml of meat slurry prepared from 2 g
cooked sausage, 1, 2, 3, 4, 5 and 6 ml of 0,5 M
sodium diethylbarbiturate aqueous solution was added
respectively, with the object of adjusting the pH
of meat slurry to 9.0.

The results given in Fig. 3 indicated that the pH
of meat slurry was adjusted to 9.0 by the addition
of 5 ml of 0,5 M sodium diethylbarbiturate aqueous
solution.

As a result of a lot of experiments, it has been
confirmed that due to the buffering capacity of
sodium diethylbarbiturate by adding 5 ml of 0,5 M
sodium diethylbarbiturate aqueous solution to 150 ml
of meat slurry to be extracted, the pH of which is
adjusted to $9.0 \pm 0,2$, even though the meat product
samples show various pH values of 5.5 to 6.5 and
contain various food additives generally used in
meat processing.

(3) Effects of pH, extracting time and stirring condition
on the amount of nitrite extracted
From 2 g cooked sausage 150 ml of meat slurry was
prepared in the same manner as described above and
the pH of which was adjusted to 9.0 by adding 5 ml
of 0,5 M sodium diethylbarbiturate aqueous solution.

For comparison, another meat slurry sample was pre-
pared following the AOAC method, namely, 5 g of the
same cooked sausage was comminuted finely and then
diluted to 300 ml with distilled water, the pH of
this meat slurry sample showed 5.9.

In both meat slurry samples, nitrite was extracted
at $80^{\circ}C$ in a water bath with occasional stirring on
one occasion and with continuous stirring on another

occasion for 30, 60, 90 and 120 minutes respectively, and the amounts of nitrite extracted were determined by the proposed method colorimetrically throughout.

According to the results represented in Fig. 4, the amounts of nitrite extracted at pH 5.9 showed much less values than those extracted at pH 9.0. In the former case the amount of nitrite extracted increased appreciably with extracting time and varied significantly with the stirring condition, while in the latter case the amount of nitrite extracted increased slightly with extracting time to a maximum value at 60 minutes and then remained unchanged until 120 minutes regardless the stirring condition.

In the former case, an appreciable amount of nitrite must have been decomposed by coexisting reducing substances at pH 5.9, i.e., at the natural pH of the cooked sausage analyzed, during the process of extraction, and the nitrite contained in the cooked sausage sample may have not completely been extracted at this pH, even though stirred continuously at 80°C for 120 minutes. Probably these are the principal reasons why the amounts of nitrite extracted at pH 5.9 by the AOAC method were much less than those extracted at pH 9.0.

Accordingly, the following is recommended as the condition of extracting nitrite.

Adjust the pH of meat slurry to be extracted to 9.0 ± 0,2, then extract nitrite at 80°C for 90 minutes with occasional shaking.

3. The procedure of the proposed method for the determination of nitrite in meat products

As described above, the pH of meat slurry to be extracted can surely be adjusted to 9.0 ± 0,2 in general by adding 5 ml of 0,5 M sodium diethyl-

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barbiturate aqueous solution.

After the extraction of nitrite at 80°C for 90 minutes
and the successive cooling, the pH of meat slurry should
be adjusted to 7.0. From the results of experiments on
this process, it has also been proved that the pH of
meat slurry extracted at pH 9.0 can surely be adjusted
to 7.0 ± 0,1 in general by the addition of 2 ml of
1 N HCl solution.

Therefore, the process of adjusting the pH of meat
slurry has extremely been simplified, and the following
procedure is proposed as a recommendable method for
the determination of nitrite in meat products.

Procedure:
Weigh 2 g of sample, homogenize with 10 ml of distilled
water for 3 minutes in a Waring blender, dilute with
destilled water to 150 ml, to adjust the pH of which to
9.0 ± 0,2 add 5 ml of 0.5 M sodium diethylbarbiturate
aqueous solution.

Extract nitrite from the meat slurry adjusted at pH 9.0
by heating at 80°C for 90 minutes with occasional sha-
king, cool with running tap water for 10 minutes and ice
water for 30 minutes respectively, add 2 ml of 1 N HCl
solution to adjust the pH of meat slurry to 7.0 ± 0,1.

Add 3 ml of 10 mM $K_3Fe(CN)_6$ solution to the meat slurry
adjusted at pH 7.0 ± 0,1, keep at 0°C for 30 minutes in
ice water, add 10 ml of 0.1 M lead acetate solution, let
stand at room temperature for 10 minutes, dilute with
distilled water to 200 ml, then filter.

To 5 ml of the clear filtrate freed from interfering
substances and proteins add 5 ml of Orange I reagent,
let the color develop by keeping at 30°C for 30 minutes,
cool with running tap water for 10 minutes, then measure
the absorbance at 479 mμ.

4. Recovery test of nitrite

In the previous work with an aqueous model system 2),
the recovery of nitrite determined by the proposed method
from the sample solutions containing various food addi-
tives at their respective levels usually employed in
meat processing was 100 %.

In the present work too, the recovery test of nitrite
from the cooked sausage samples containing various food
additives at their respective levels usually employed
in meat processing was performed in the following way.

To each 150 ml of meat slurry prepared from 2 g of
cooked sausage sample and adjusted at pH 9.0 ± 0.2,
2 ml of each food additive solution containing each food
additive at the concentration given in Table 1, together
with 50, 100 and 150 ppm of sodium nitrite respectively,
was added, then the amount of nitrite was determined by
the proposed method described just above.

In this case too, the average recovery of nitrite from
the cooked sausage samples containing various food
additives was found to be 100 % with extremes of 99.6 to
100,3 %.

5. Comparison between the findings for nitrite determined
by the AOAC method and those determined by the proposed
method

Using the very same sample, the amount of nitrite in
cooked sausage was determined by the AOAC method and
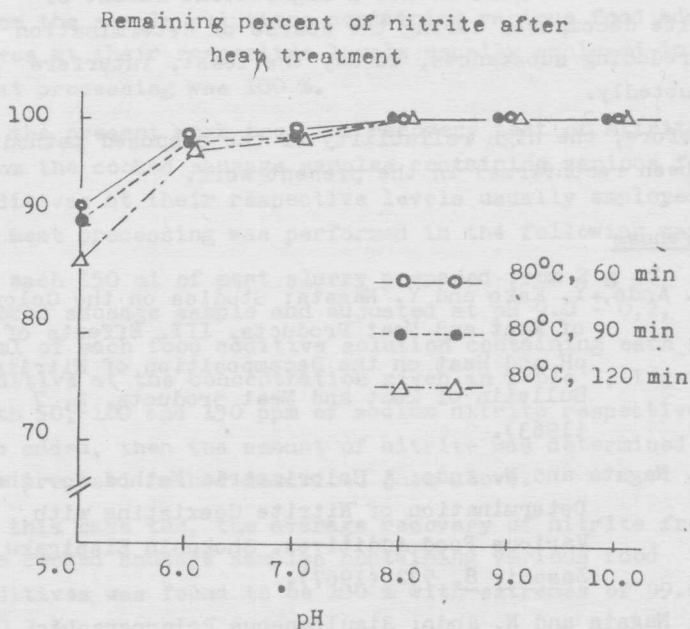the proposed method, respectively.

According to the results represented in Tabel 2, the
amount of nitrite determined by the AOAC method was
considerably less than that determined by the proposed
method. Furthermore, the addition of ascorbate resulted
in a significant decrease in the amount of nitrite
found in the AOAC method, while the addition of ascorba-
te did not interfere at all in the proposed method and

the amount of nitrite found remained quite unchanged.

From the above results, it has evidently been demonstrated that in the AOAC method a significant amount of nitrite decomposes during the course of determination and reducing substances, to say the least, interfere undoubtedly.

Therefore, the high reliability of the proposed method has been reconfirmed in the present work.

References

1. N. Ando, Y. Kako and Y. Nagata: Studies on the Color of Meat and Meat Products. III. Effects of pH and Heat on the Decomposition of Nitrite. Bulletin of Meat and Meat products, 2, 7 (1963).

2. Y. Nagata and N. Ando: A Colorimetric Method for the Determination of Nitrite Coexisting with Various Food Additives. Shokuhin Eiseigaku Zasshi, 8, 532 (1967).

3. Y. Nagata and N. Ando: Simultaneous Polarographic Determination of Nitrite and Reduced Form Ascorbate, 12, 424 (1966 - 1967)

4. Methods of Analysis, A.O.A.C., 10th. Ed., p. 347 (1965).

5. R. Grau und A. Mirna: Über die Bestimmung von Nitrit, Nitrat und Kochsalz in Fleischwaren und Laken. Z. Anal. Chem., 158, 182 (1957).

6. M.J. Follett and P.W. Ratcliff: Determination of Nitrite and Nitrate in Meat Products. J. Sci. Food Agr., 14, 138 (1963).

Remaining percent of nitrite after heat treatment

○—○ 80°C, 60 min
●---● 80°C, 90 min
△—△ 80°C, 120 min

100
90
80
70

5.0　6.0　7.0　8.0　9.0　10.0
pH

Fig. 1 Effects of pH and heat treatment on the decomposition of nitrite coexisting with reducing substances
(Each sample solution contains 0,002 % $NaNO_2$, 0,02 % sodium ascorbate and 0,02 % cysteine.)
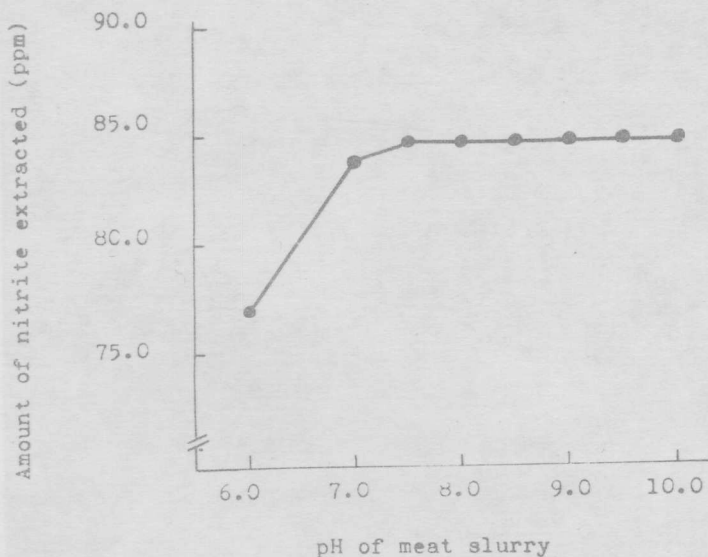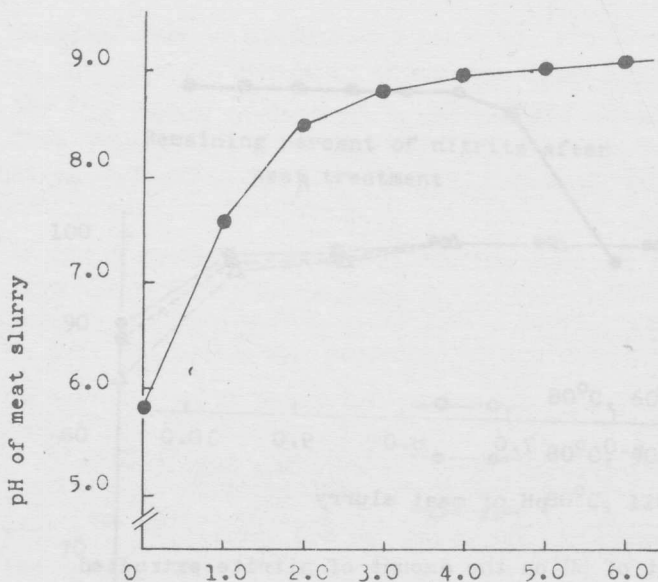
Fig. 2 Effect of pH on the amount of nitrite extracted
from cooked sausage

(To each 150 ml of meat slurry prepared from 2 g
sausage sample, 5 ml of 0,5 M sodium diethylbarbi-
turate aqueous solution was added and the pH of
which was adjusted with 1 N HCl or 1 N NaOH to
6.0, 7.0, 8.0, 9.0 and 10.0 respectively, from each
of them nitrite was extracted at 80°C for 90 minu-
tes, then the amounts of nitrite extracted were
determined by the proposed method colorimetrically.)

0.5 M sodium diethylbarbiturate aqueous
solution added (ml).

Fig. 3   Effect of sodium diethylbarbiturate on the pH
of meat slurry

(To each 150 ml of meat slurry prepared from 2 g
sausage sample, varying volumes of 0,5 M sodium
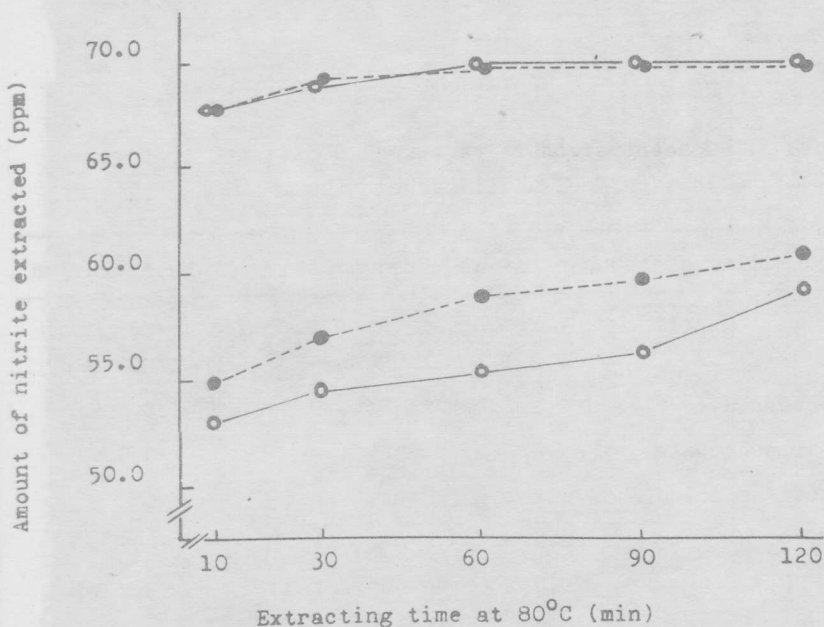diethylbarbiturate aqueous solution were added.)

Fig. 4 Effects of pH, extracting time and stirring condition
on the amount of nitrite extracted

(The meat slurry of pH 5.9 was prepared by diluting 5 g of
finely comminuted sausage sample with distilled water to
300 ml.
The meat slurry of pH 9.0 was prepared by homogenizing 2 g of
sausage sample with 10 ml of distilled water, diluting with
distilled water to 150 ml and then adjusting the pH of which
with 5 ml. of 0.5 M sodium diethylbarbiturate aqueous solu-
tion to 9.0.
The amounts of nitrite extracted were determined by the pro-
posed method throughout.)

—O—O— stirred occasionally
—●—●— stirred continuously

Table 1. Concentration of each food additive in each food additive solution

Food Additive	Concentration	Food additive	Concentration
Na CL	3 %	$Na_2 H_2 P_2 O_7$	0,5 %
KNO_3	0,2 %	$Na_4 P_2 O_7$	0,5 %
Na-ascorbate	0,2 %	$Na_5 P_3 O_{10}$	0,5 %
Na-isoascorbate	0,2 %	$Na_6 P_6 O_{18}$	0,5 %
Nicotinamide	0,2 %	Sorbic acid	0,2 %
$NaH_2 PO_4$	0,5 %	K-sorbate	0,2 %
$Na_2 HPO_4$	0,5 %	Na-sorbate	0,2 %
$Na_3 PO_4$	0,5 %	AF 2[+]	0,025 %

Each food additive solution was prepared by dissolving each food additive in a veronal buffer solution of pH 6.0 to give a respectively desired final concentration.

+ A mixture of 1 part of AF 2 and 49 parts of soluble starch. Accordingly, the AF 2 solution contains 5 ppm of AF 2. AF 2, i.e., 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, is a new preservative permitted in Japan.

Table 2   Comparison between the findings for nitrite determined by the AOAC method and those determined by the proposed method

Method	Addition of Na-ascorbate [b]	pH of meat slurry [a]		$NaNO_2$ found [c]	
		Before heat extraction	After heat extraction	ppm	Index number
AOAC method	-	5,88	5,86	50,2	100,0
	+	5,87	5,86	46,8	93,3
Proposed method	-	8,96	8,90	62,1	123,7
	+	8,98	8,90	62,1	123,7

a) In the AOAC method, 300 ml of meat slurry was prepared from 5 g cooked sausage sample finely comminuted by diluting with distilled water. In the proposed method, 150 ml of meat slurry was prepared from 2 g of cooked sausage sample homogenized with 10 ml of distilled water by diluting with distilled water, and the pH of which was adjusted to 9.0 ± 0.2.

b) To each meat slurry just before heat extraction, 0.2 % sodium ascorbate aqueous solution was added at the rate of 1 ml per gram sausage sample used, and from which nitrite was extracted in its respective way.

c) In the AOAC method, the amount of nitrite extracted was determined by the AOAC method. In the proposed method, the amount of nitrite extracted was determined by the proposed method.

cette observation a conduit, en toute logique, les professionnels et les chercheurs à préconiser l'emploi de sel nitrité en remplacement du sel nitraté. La législation française, après avoir longtemps refusé d'admettre ce procédé de salage en raison de la toxicité bien connue des nitrites, a fini par l'accepter (A.M. du 15 septembre 1964) en le soumettant à des conditions très strictes pour éviter tout risque d'intoxication accidentelle du consommateur: elle a en particulier fixé à 150 mg par kilogramme la teneur en nitrite de sodium dans les denrées livrées à la consomation.

Si, de l'avis de nombreux experts, cette technique va à l'encontre de la qualité gastronomique des produits finis, elle ne manque pas de présenter un certain nombre d'avantages dont les plus couramment admis sont:

a) le gain de temps car, d'une part le nitrite de sodium pénètre plus rapidement dans la viande que le sel et le nitrate de potassium, d'autre part on supprime les longs délais (jusqu'à 1 mois à basse température) de la phase bactériologique de réduction des nitrates en nitrites.

b) l'amélioration de la qualité des produits finis, du fait de la suppression des phénoménes bactériologiques toujours difficiles à controler et, partant, souvent responsables d'accidents de fabrication.

c) la diminution des risques de toxicité pour le consommateur, par suite de la possibilité de controler les quantités résiduelles du nitrite dans les produits finis.

C'est en effet une des critiques majeures de nombreux experts de la santé publique à l'encontre de la salaison au sel nitraté: il n'est pas possible de controler la quantité de nitrate de potassium résiduel dans les produits finis et le consommateur risque d'ingérer ainsi des quantités de ce corps dangereuses pour sa santé. Cela tient à ce que la réduction bactériologique du nitrate en nitrite est toujours incomplète (30 à 40 p. 100 au maximum); il