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COMPARISON OF A NEW HPLC-UV METHOD FOR THE DETERMINATION OF NITRITE AND NITRATE IN CURED MEATS WITH THE ENZYMATIC METHOD

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Background:

Combinations of nitrite and nitrate salts are commonly used as additives for curing meat products like sausages, cooked ham and different kinds of raw sausages. Besides the principal function of preventing food poisoning from *Clostridium botulinum* [1], they give cured meats their characteristic colour and organoleptic properties [2]. However, nitrite can lead to methaemoglobinaemia or to the formation of the potent nitrosamine carcinogens [3]. Nitrate is less toxic then nitrite, but can be converted by microbial reduction to nitrite. Due to this toxicologic fact the maximum permitted levels of nitrite and nitrate in cured meats are controlled by common legislation in the European community [4]. In Germany, two official standard methods for the determination of nitite and nitrate exist [5]: the first is the classical cadmium-reduction griess method; the second is based on the reduction of nitrate to nitrite by the enzyme nitratereductase. In addition to these official methods, several analytical HPLC [2, 6] and IC [7] methods with anion detection by UV absorption, electrochemical detection [8], or others, have been developed during the recent years. The use of such chromatographic methods is very attractive as they are more rapid, can be automated, and when the interference by the sample matrix is eliminated, more sensitive and selective then the others.

Objectives:

The aim of our work was to establish an easy-to-handle and inexpensive method for the simultaneous determination of nitrite and nitrate which doesn't need time and work consuming clean-up steps or specialised equipment.

Methods:

Materials : Samples of cured meats were purchased as prepacked products from local stores and were stored as recommended in a refrigerator at 4°C until analysed. All chemicals were of analytical grade; deionised water was used.

Instrumentation : A Hewlett Packard (Waldbronn, Germany) HP 1100 chromatography system consisting of an isocratic pump, autosampler, thermostated column compartment and a variable UV/VIS wavelength detector was used. The analytical PRP-X100 anion exchange column was obtained from Hamilton (Reno, USA). Chromatographic data were analysed with a Chromatopac C-R5A (Shimadzu, Kyoto, Japan). For the enzymic analysis we used a DU 64 spectralphotometer from Beckmann (Munich, Germany). Meat samples were mixed and homogenised using a Moulinette, (Moulinex, Solinen, Germany) and a Ultra Turrax (Janke and Kunkel, Staufen, Germany)

Sample preparation: The samples were cut into small pieces using a kitchen knife and then minced with a Moulinette blender. 8 g and 10 g aliquots of the finely comminuted samples were weighed and immediately treated with 10 mL saturated borax solution (50 g/L) and 80 mL water heated to 80°C. Aqueous extracts were prepared by homogenising the mixture at 13,500 rpm for 1 minute with the Ultra Turrax. The samples were then heated in a boiling water bath for 15 minutes with repeated shaking. After the samples cooled down to room temperature they were transferred into 200 mL volumetric flasks. 2 mL Carrez I ($K_2[Fe(CN_6)] \cdot 3H_2O$, 150 g/L) and 2 mL Carrez II ($Zn(CH_3COO)_2 \cdot 2H_2O$, 230 g/L) were added successively with shaking and the final volume was made up to 200 mL with water. After 30 minutes the samples were filtered through a Schleicher & Schuell No. 595 ½ and then through a 0,22 µm nylon filter disk. The same aqueous extract was used for both analytical methods.

Chromatographic conditions :

The column was eluted at a flow of 1 mL/minute with a Tris buffer at pH 8.6 which was prepared as follows: 3.8 mM Tris and 70 mM sodium chloride were dissolved in water containing 15 % (v/v) acetonitrile (ACN), and were adjusted with 2 M hydrochloric acid to pH 8.6. The temperature of the column compartment was 25°C; the injected volume of sample and standard solution was 20 µL. Under these conditions nitrite was detected after 2.7 minutes at 225 nm and nitrate after 5.2 minutes at 220 nm, as shown in figure 1. For calibration, a nitrite/nitrate stock solution of 100 mg/L was prepared by accurately weighing appropiate amounts of sodium nitrite and potassium nitrate into a 200 mL volumetric flask, then dissolving and makeing it up with water. The stock soultion was diluted to 10 mg/L and finally working standards were prepared by diluting this standard solution and treating them like the samples with borax solution, Carrez I and II. Calibration curves were made by plotting the peak areas against the concentrations of the standards injected. Triplicate injections at the following levels were made : 0.2; 0.5; 0.7; 1.0; 2.0; 3.0; 4.0 and 5.0 mg/L

Enzymic determination : A testkit from Boehringer (Mannheim, Germany) was used. For the measurement of nitrate, 400 µL reaction mixture (NADPH, FAD) and 800 µL sample solution were pipetted into cuvettes. The blank absorbance of this solution was measured and 20 µL of nitrate reductase was added. After standing for 60 minutes, 1220 µL colour reagent (reagent A consisted of 600 mg sulfanilamide, 25 mL concentrated HCl and the whole made up with water to 100 mL, mixed in the same aliquots with reagent B 100 mg N-(1-naphtyl)ethylenediaminedihydrochloride in 100 mL water) was added. After standing in the dark for 30 minutes, the absorbance was measured at 540 nm. To determine the nitrite, 800 µL of sample solution was mixed with 800 µL of colour reagent and after 30 minutes the absorbance at 540 nm was also measured. For the calibration, two different stock solutions of nitrite and nitrate were made and final working standards with 0.15; 0.30; 0.45; 0.60 mg/L for nitrate and 0.1; 0.2; 0.3; 0.4 mg/L for nitrite, were prepared. The standard solutions were also treated in the same way as the samples.

Results and discussions:

The results we obtained by this HPLC method, in comparison with the enzymic German standard method, are shown in Table 1. The data show that there is good agreement between both techniques. The difference in the nitrite/nitrate content between the methods show that almost all pairs of results are within the comparability range of 8 mg/kg, which is given for the enzymic method when the same sample is analysed at different laboratories by different persons and at different times.

Table 1:

	Cooked ham				Bologna type sausage				Raw cured meat products*			
	$NaNO_2$ [mg/kg]		KNO_3 [mg/kg]		$NaNO_2$ [mg/kg]		KNO_3 [mg/kg]		$NaNO_2$ [mg/kg]		KNO_3 [mg/kg]	
Sample	Enzymic	HPLC	Enzymic	HPLC	Enzymic	HPLC	Enzymic	HPLC	Enzymic	HPLC	Enzymic	HPLC
1	27	27	19	20	15	17	58	60	5	10	125	120
2	16	20	27	27	14	14	38	38	15	9	72	73
3	29	30	21	20	21	24	37	31	114	115	155	160
4	5	5	8	6	15	16	56	55	7	8	45	43
5	5	7	185	184	5	7	17	14	33	31	69	70
6	37	44	16	18	28	27	44	44	9	5	173	172
7	7	5	18	15	34	31	27	26	7	5	230	218
8	35	38	23	20	32	31	53	55	9	3	135	135
9	9	9	16	19	13	13	42	39	5	2	10	7
10	13	13	13	13	6	13	47	45	8	6	14	16

* Sample numbers 1 to 5 are raw ham, numbers 6 to 10 are dry sausage

To prevent decomposition of nitrite during analysis, we adjusted the pH of the mobile phase to 8.6, which is in the range of the sample media after the addition of saturated borax solution. As our aim in this study was to establish a fast and inexpensive method for the determination of nitite and nitrate, we used only the Carrez reagents for protein precipitation during the sample clean-up step. This could lead to interferences between nitrite and nitrate ions with co-eluting sample matrix, which could be removed by the use of cyclohexyl SPE cartridges [2]. We found that the addition of acetonitrile in the mobile phase also moves the co-eluting sample matrix to an earlier retention time. The retention times of nitrite and nitrate were not influenced so much by the addition of 0 %, 15 %, 22,5 % or 30 % acetonitrile because the concentration of Tris and NaCl were the same. As shown in figure 2, the use of 15% acetonitrile shows a great advantage during the analysis of cooked ham and Bologna type sausage samples. For the raw cured meat products a higher content of acetonitrile, 22,5 % or 30 %, was used (see figure 3).

After about 300 injections of different cured meat samples, we didn't find a noticeable change in the retention times of nitrite and nitrate, or in an increasing pressure of the HPLC system. So we conclude that there is no irreversal adsorbance of sample matrix in the HPLC column. Because of the use of acetonitrile, no further sample clean-up step is needed and no risk of microbiological growth is expected. This could save time, work and money.

Conclusions:

Our study describes a simple and inexpensive HPLC-UV method for the determination of nitrite and nitrate in different cured meat samples. The results obtained by this method and the results of the enzymic method are in good agreement. If our further studies also show good accuracy and reproducibility, we expect that this method could be a useful alternative to the German standard methods.

Figure 1: Nitrite and nitrate standards

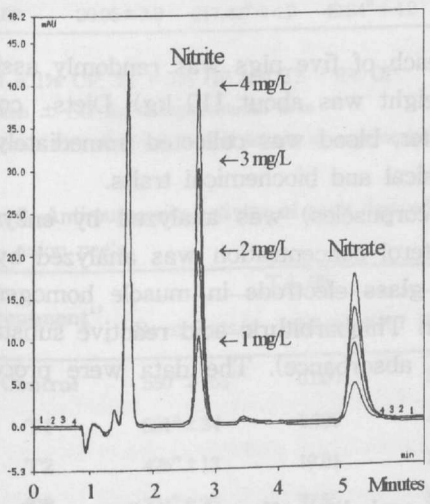


Figure 2: Chromatogram of cooked ham

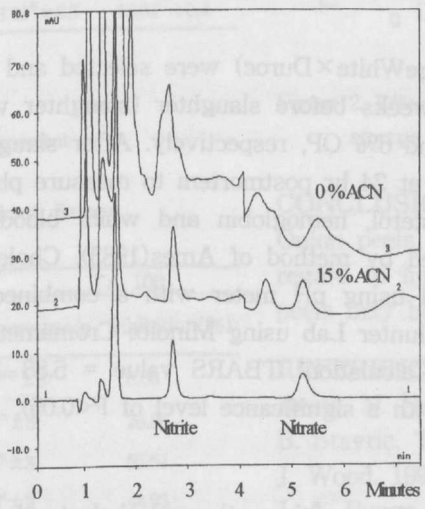
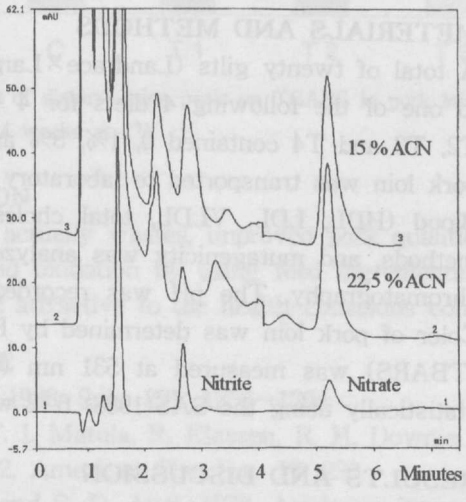


Figure 3: Chromatogram of salami



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