FULL AUTOMATED ROBOTIC METHOD FOR THE DETERMINATION OF CHLORIDE, NITRITE AND NITRATE IN CURED MEAT PRODUCTS.

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1. Background

Curing is one of the most used technologies in meat manufacturing. The different curing modes (namely dry curing, wet curing, injection curing [1]) are based on the same principle: addition to the fresh product of a mixture of sodium chloride and either nitrite or nitrite plus nitrate salts. Although the use of these curing agents involves some minor disadvantages, the advantages are so overhelming [2] that no alternatives to their use are available at present. In the case of nitrite and nitrate, some of the most remarkable beneficial aspects of the curing process [3] are as follows: appearance of a red colour due to a reaction between muscular hemoglobin and the nitric oxide generated in nitrite reduction; reaction of nitrite and different compounds such as sugars, alcohols, amines, etc. to yield products which contribute to the typical aroma of meat cured products; antioxidant effect on the lipids rancidity; antimicrobial activity, which hinders the growing of pathogenic microorganisms, such as *C. Botulinum*. The negative effects of the curing anions on health only appear in products with an excess of these agents [4]. Some of the well-known undesirable effects are: the oxidant action of nitrite on the hemoglobin-Fe(II) complex, which can produce poisoning symptoms; formation of N-nitrosocompounds in the presence of both nitrosamine formers and acid pH; and the toxic action of nitrate, not *perse* but due to reduction to nitrite. For these reasons, the routine analysis of nitrite and nitrate is a common practice in meat industries in order to keep their level in the products within a range which enables the development of the beneficial effects without appearance of the toxic aspects. On the other hand, the presence of sodium chloride in the curing process has as main advantages its protective effect which hinders or makes difficult germs development by decreasing water activity; its conserving action by both potentiating the effects of other preservant agents and inhibiting the fungii and yeasts proliferation; and increasing the flavour. High salt concentration in cured products result in rejection by the consumers.

2. Objetive

We have developed a fully automated method based on the coupling of a robotic station for sample weighing and pretreatment and a continuous flow system for derivatisation and detection in order to help big meat industries in the development of the routine determination of these three more commonly determined parameters.

3. Methods

Manual sample pretreatment and determination

Sample pretreatment. The meat sample is grinded and homogenised. A representative amount (ca. 10 g) of sample is weighed (within 1 mg precision) and introduced into a 250 ml Erlenmeyer flask where 5 ml of 5% di-sodium tetraborate decahydrate solution and about 150 ml of distilled water at 60 °C are added. The resultant suspension is maintained warm and stirred for 30 min, after which, it is transferred to a 200 ml volumetric flask and 2 ml of each Carrez reagent are added. The suspension is stirred, let cool down and made to volume. Then, it is filtered and the filtrate transferred to a 250 ml Erlenmeyer flask.

Determination of nitrite. Between 5 and 10 ml of filtrate are taken and introduced into a 25 ml volumetric flask. Distilled water until ca. 20 ml and 1 ml of Zambelli reagent are added and the solution stirred, then stood at room temperature for ten minutes. After this, 1 ml of an ammonia concentrated solution is added and made to volume with distilled water. It is let cooled down for ten minutes more and the absorbance of the solution is then measured at 436 nm.

Determination of nitrate. 20 ml of the filtrate are transferred to a 25 ml Erlenmeyer flask. 5 ml of a 5% NH_4OH solution are added and the mixture is heated until getting a soft boiling and then it is passed through the cadmium column. The eluate is taken in a 100 ml volumetric flask. The column is rinsed for three minutes with hot water and the liquid is added to the volumetric flask which is made to volume. The nitrite obtained after this treatment is determined in the solution by the previously described method.

Determination of chloride. 10 ml of the filtrate, ca. 100 ml of distilled water and some drops of a 10% K_2CrO_4 solution are added to a 250 ml Erlenmeyer flask. This solution is titrated with a 0.1N $AgNO_3$ solution until appearance of a red colour.

Proposed procedure

Sample pretreatment. The robot gets the big-size object all-purpose hand (BSOAP), catches a 250 ml fleaker from the rack and places it on the balance plate and tares it. The sample is grinded using a coffee grinder connected to an AC output (AC2) of the power and event controller (PEC). After this, the robot takes the small-size object all purpose hand (SSOAP) and uncovers the grinder, turns its hand 180° to make available the dispenser hand (SD), which is placed over the grinder, and introduces into it in order to take an amount of sample, which is added into the fleaker placed on the balance plate. If the sample weight is lower than 10 g, the robot repeats the operation as many times as necessary until the amount of sample is higher than 10 g (i.e. between 10.0 and 12.0 g). The controller collects the weight-datum, with precision of 1 mg, and the robot takes the fleaker out from the balance, sets it under the dilute & dissolve dispenser, adds 5 ml of 5% di-sodium tetraborate decahydrate solution inside the fleaker and then places it on the stirrer. It takes the distilled water dispenser, sets it over the fleaker and adds 150 ml which are aspirated from a reservoir in a thermostated bath at 60 °C. The controller drives the AC output (AC1) of the PEC, thus the stirrer is turned on and the sample stirred for 30 min maintaining the temperature at 60 °C until the time is over. The robot carries the fleaker from the stirrer to the dilute & dissolve module where 2 ml of each Carrez reagent are added and then returns it to the stirrer where the mixture is stirred for 20 s. Now, the robot catches the fleaker and places it in the fleakers rack. Once the solution is cooled down the robot places the fleaker on the balance plate, takes the water dispenser and adds distilled water until the weight is 200 g. Then, the robot returns the fleaker to the stirrer in order to homogenise the solution, takes the filter, introduces it into the solution and acts the master laboratory station 2 (MLS2) syringe, connected to the filter. Now, the syringe is filled up with filtered solution and the controller, after changing the position of the selection valve, empties the syringe content into a clean fleaker. This operation is repeated twice after which the controller does not change the position of the valve once (the liquid goes back by the same way that it has entered) in order to rinse the filter from sticked particles. The filtered solution is aspirated by means of the aspiration probe connected to the injector located in the flow injection manifold with the aid of the peristaltic pump.

The hydrodynamic system (FI configuration) for the *determination of nitrite and nitrate* is shown in Fig. 1B. Valve SV lets the sample to merge directly with the reagents for the nitrite determination (filling position). In the inject position the sample passes through the reduction column before mixing with the reagents. The original nitrite and the nitrite resulting from the reduction of the nitrate are thus determinated. The concentration of nitrate is calculated by difference.

The continuous manifold for the determination of nitrate is also used for the *chloride determination.* The sulphanilamide and N-1-naphthylethylenediamine solutions are exchanged by distilled water and chloride reagent solution, respectively. The carrier is distilled water in all instances.

Calibration. It is accomplished by running a calibration curve with 7 standards every week. 3 standards are inserted every day in order to correct the slope, if necessary.

Overall procedure. Once the filtrate has been obtained by the robot, it is aspirated by means of the aspiration probe connected to the injection valve of the flow injection manifold with the aid of the peristaltic pump. First, the nitrite is determined, then the nitrate, in both cases by interpolation of the peak absorbance of the sample in the corresponding calibration line stored in the computer, and finally the chloride is determined using electronic dilution for collection and data treatment.

Computer program for electronic dilution. Two main reasons made the use of a computer program necessary for chloride determination: 1) the wide determination range of this analyte (between 3 and 18% w/w) which makes mandatory signal measurement at times longer than the residence time without significant errors; 2) management of the huge number of data obtained in routine analysis of large series of samples (up to 400 sample/day).

Signal-time data are collected by the computer a preset intervals from the maximum of the peak. These data are stored either for individual samples or batch of samples. In the former case the computer works like a recorder. In the latter case the time at which the absorbance is measured is selected when the data from all the samples have been acquired. Depending on the concentration of the target sample, the time selected can be that corresponding to the maximum or a larger time in the tail of the fiagram where the absorbance is lower due to a higher dispersion. Once the time is selected, the computer provides the absorbance signal, which is imported to a LOTUS chart from which the calibration line corresponding to the selected time is run and the absorbance of the sample interpolated in it.

4. Results and discussion

Chemical systems

The determination of nitrite is based on the Griess' reaction, adopting the Shin's modification [9] in order to avoid the manipulation of carcinogenic reagents. The determina-tion of nitrate is based on the same derivatisation reaction after reduction to nitrite. The determination of chloride is based on displacement of thiocyanate in the mercury-thiocyanate complex. Then, the displaced thiocyanate forms a red complex with ferric ion, which is monitored at 480 nm. As the overall process consists of two parts (discontinuous or robotic and continuous or FI), the optimisation of each was accomplished separately.

Discontinuous or robotic stage: sample pretreatment

A computer program was developed for the control of the different unitary operations (UOs) to be carried out by the robotic station. The performance of both the program and the different UOs was then checked.

In order to obtain a grinded sample suitable for being handled by the robot (not sticky mass but divided in small particles) the grinding time must be short (about 10 s is sufficient).

The solutions were made to weight rather than to volume as the robot was a sensorless device.

Determination step

Methods for the determination of nitrite, nitrate and chloride by flow techniques described in the literature are abundant. The ruggedness of the method for chloride is guaranteed by its routine use since 1960 in segmented-flow analysers and since 1975 in FI. As the aim of this research was focused on the overall automation rather than to development of new determination methods, some variables as reagent concentration, diameter of the tubing system, length of the reactors and geometry of the reduction column were not optimised and the values in previous works developed by our research team [10-12] were used (see Table 1).

Features of the methods

Nitrite and nitrate determination. The features of this method require to run three calibration lines. One for nitrate and two for nitrite (with and without passage of the sample through the reduction minicolumn). Series of solutions were prepared from solutions of both anions within a wide range of concentrations and subjected to the sample pretreatment to minimise the matrix effects. Linear ranges between 0.1-15 mg l^{-1} for nitrite and between 0.5 and 30 mg l^{-1} for nitrate were obtained. These ranges are appropriate for the determination of these anions in meat. The RSD obtained for 11 samples of 5 mg l^{-1} injected in triplicate was lower than 1.5 % in all instances. The sampling frequency was 30 h^{-1}.

Chloride determination. The characteristics of this system makes necessary to run only one calibration line. The socalled electronic dilution was used in order to adapt the method to the usual wide range of concentrations of this anion in meat (between 3 and 18% (w/w). The data were acquired and processed using a computer program designed in our laboratory. Standards were prepared in a wide range of concentrations.

Application of the method to meat samples

The proposed method was validated by applying it to 10 samples of fresh and cured meat from Navidul S.A. The content of the analytes in the samples was also determined by the manual method.

4. Conclusions

A completely automated method for the determination of nitrite, nitrate and chloride in cured meat based on the coupling of discontinuous (robotic)/continuous (flow injection) automated alternatives is proposed.

The assembly works in an unattended fashion thus eliminating human intervention with the subsequent decrease of the overload creates in routine laboratories by the determination of these common parameters.

The method also allows a reduction of the analyses time as the conventional methods for these analytes involve a number of steps which are deleted in the automated counterpart here reported. This shortening in the time required for the overall process is mainly due to the faster developement of the derivatisation/monitoring step, wich takes place in the non-equilibrium conditions characteristic of FI methods. A decrease in the time required for this step from 20 to 3 min is thus achieved. The time for the development of the robotic preliminary operations is similar than that required by the conventional method, the most significant advantage of its implementation being the elimination of users intervention wich makes feasible a 24 hours working-day.

5. Literature

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Table 1. Optimum values of variables for the determination of NO_2^-, NO_3^- and Cl^-

Parameter	Optimum value
Injection volume (NO_2^-/NO_3^-), µl	100
Injection volume (Cl^-), µl	10
Flow-rate, ml min^{-1}	1.1
R1 length, cm	15
R2 length, cm	200
Column length, cm	8.5
Column diameter, mm	3