Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 92 (2005) 373–379
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Determination of niacin in fresh and dry cured pork products by ion chromatography: experimental design approach for the optimisation of nicotinic acid separation

G. Saccani [a,*], E. Tanzi [a], S. Mallozzi [a], S. Cavalli [b]

[a] *Stazione Sperimentale per l'Industria delle Conserve Alimentari, Viale Tanara 31/A, 43100 Parma, PR, Italy*
[b] *Dionex Laboratorio Applicazioni, Via Tulipani 1, 20090 Pieve Emanuele, MI, Italy*

Received 26 January 2004; received in revised form 7 October 2004; accepted 18 October 2004

Abstract

A simple and robust method to determine the niacin level in fresh and dry-cured pork products by ion chromatography is reported. The analytical procedure includes an acidic hydrolysis to free all the bound forms (NAD and NADP) and to convert all the niacin into nicotinic acid, after that the chromatographic separation of the acid form is performed by a cation-exchange column and UV detection (262 nm).

The evidence of several interferences that did not allow a good resolution of the nicotinic acid peak in fresh meat matrix led us to improve the resolution by means of statistical optimisation. A three level factorial design and response surface methodology were applied to optimise the chromatographic conditions (mobile phases compositions) and to assess the robustness of the method.

Analytical performances of the optimised method were investigated in terms of linearity, limit of detection (LOD) and precision.

The complete analytical procedure to extract and determine the nicotinic acid in meat was validated by IC-MS.

Keywords: Niacin; Nicotinic acid; Meat products; Response surface methodology; Ion chromatography

1. Introduction

Nutritional studies, designed food label information and food quality control need simple, accurate and robust analytical procedures especially to determine vitamin levels.

Meat and meat products are one of the main sources of the B group water-soluble vitamins and in particular of niacin (nicotinic acid and nicotinamide), which plays an essential role in a large number of biological redox reactions as a part of nucleotide coenzyme NAD and NADP (Ball, 1994; http://www.nutrition.org/nutinfo/content/niac.shtml).

A recent opinion expressed by the European Scientific Committee on Food (2002), available at the web page http://www.europa.eu.int/comm/food/fs/sc/scf/out80j_en.pdf, establishes an upper daily intake of niacin to avoid undesirable effects and set the tolerable upper intake level of niacin to 30 mg/die for adults on the base of flushing reaction produced by a direct intake of nicotinic acid.

For its antioxidant properties, niacin is known to minimise the oxidative reactions in foods and, especially, in meat products where reduces the speed of myoglobine oxidation and prolongs the red fresh colour in minced meat products. Up until the 1980s in USA and also in Germany it was possible to exploit this property

* Corresponding author. Tel.: +39 0521 795219; fax: +39 0521 771829.
E-mail address: relyfood@ssica.it (G. Saccani).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.10.007

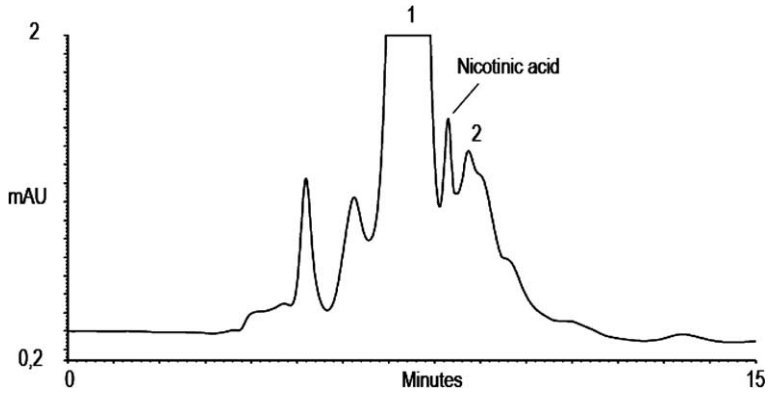

Fig. 1. Chromatographic separation of nicotinic acid in a fresh pork sausage sample after acidic hydrolysis (d:10). Column: OmniPac PCX-500 2 mm, eluent: 80 mM HCOOH – 50 mM NH_4COOH – 5% acetonitrile, flow rate: 250 μL/min, column temperature: 40 °C, detector: UV 262 nm. In this analytical condition the nicotinic acid is strictly close between two interferences substances (peaks n.1 and n.2).

by adding restricted quantities of niacin in meat products, especially in minced ones, such as hamburgers or fresh sausages, with the aim to enhance fresh meat colour and to extend the shelf-life of pre-packed fresh meat products. (Bertling & Tietz, 1978; Kendrick & Watts, 1969) Because niacin is not allowed as antioxidant in food and it is not included in the positive list of legal additives in European Community (Directive 95/2/EEC), neither as acid nor as amide, it is important to develop a simple and straightforward method to evaluate niacin in meat products and to check meat samples, fresh and processed, for niacin occurrence.

Over the last few years much attention has been paid in developing chromatographic and electrophoretic methods for niacin determination in food and ion-pair, reverse phase and ion-exchange chromatographic methods are widely reported (Balschukat & Kress, 1990; Hamano, Mitsuhashi, Aoki, Yamamoto, & Oji, 1988; Hirayama & Maruyama, 1991; Polesello & Rizzolo, 1986; Rizzolo & Polesello, 1992, 1998; Takatsuki, Suzuki, Sato, Sakai, & Ushizawa, 1987; Tyler & Genzale, 1990; Ward & Trenerry, 1997).

Since in muscle tissue niacin is present in bound (NAD and NADP) and free forms (acid and amide), an accurate determination requires an hydrolysis procedure, usually followed by a sample clean-up to eliminate the interferences before HPLC analysis or/and derivatization procedure for improving selectivity and sensitivity. (Chase, Landen, Soliman, & Eitenmiller, 1993; Holasova & Maskova, 2001; Vidal-Valverde & Reche, 1991; Ward & Trenerry, 1997).

The lack of fluorescent moieties and the low specificity of UV detection at 262 nm do not allow an accurate determination of niacin in food matrices, due to interferences derived from the standard hydrolysis procedure. To overcome this critical problem and to improve the selectivity of HPLC most Authors introduced time-consuming sample clean-up or derivatization procedures (Krishnan, Mahmud, & Matthees, 1999; LaCroix & Wolf, 2001; Lahély, Bergaentzlè, & Hasselmann, 1999; Tyler & Genzale, 1990).

During a method setup, we verified that the selectivity and repeatability problems, connected with widely used ion-pair or reverse-phase methods, could be overcome by ion chromatography which was able to separate simultaneously nicotinic acid and nicotinamide after a rapid sample preparation and without any sample clean-up procedure.

However the evidence of interferences, especially in fresh sausage samples, after standard acidic digestion (Fig. 1) led us to improve the method selectivity optimising the nicotinic acid resolution from the main interferences (peaks n.1 and n.2 in the figure) with a statistical approach, instead of introducing complex and time-consuming sample clean-up or derivatisation procedures.

The aim of our work was to develop a simple and accurate chromatographic method as a suitable tool for nutritional and food quality control of niacin in fresh and processed meat, particularly in pork meat, the most used to prepare cooked and dry-cured meat products.

In addition to UV detection, a MS spectrophotometer let us to assess the accuracy of nicotinic acid determination and the effectiveness of hydrolysis procedure.

2. Experimental

2.1. Chemicals and reagents

Nicotinic acid and nicotinamide were purchased from Sigma–Aldrich (Milan, Italy). Analytical reagent grade formic acid (96%), ammonium formate and hydrochloric acid were from Aldrich Chemical Company (Steinheim, Germany).

Acetonitrile and methanol of HPLC gradient-grade was purchased from Merck (Darmstadt, Germany). Water for chromatography was purified (18 M cm^{-1}) with a Milli-Q® system (Millipore, Bedford, MA, USA).

Standard solutions of nicotinic acid and nicotinamide were prepared in 0.01 N HCl. All stock solutions (1 mg ml^{-1}) were stored at 4 °C and protected from light. Working solutions for calibration were suitably diluted just before the HPLC analysis.

2.2. Equipment and chromatographic condition

Chromatographic analyses were performed on a DX-500 Ion Chromatograph (Dionex, Sunnyvale CA, USA) equipped with a GP40 Gradient Pump, a PDA100 photodiode array detector and an AS3500 Autosampler. A 250 × 2 mm i.d. OmniPac® PCX 500 (Dionex, Sunnyvale CA, USA) microbore column (macroporous cross-linked ethylvinylbenzene-divinylbenzene copolymer, functionalized with radiation grafted sulphonic acid cation exchange groups) was used with acetonitrile/formic acid/sodium formate based eluent. The column temperature was set at 40 °C and all samples were filtered through 0.45-μm filters. Dionex Chromeleon® 6.40 chromatography software allowed to control data collection and the operation of all components in the system.

Chromatographic conditions are summarized in Table 1.

2.3. MS detection

Peak confirmation was obtained through MS detection carried out by a single stage quadrupole detector with enhanced low-mass option (MSQ ELMO, Dionex, Sunnyvale, CA, USA). The MS was operated in the positive electrospray (ESI+) ionization mode at 3.0 kV. Probe temperature was set at 350 °C, cone voltage was 50 V.

The protonated molecular ions $[M + H]^+$ of nicotinic acid and nicotinamide were detected at a mass-to-charge ratio (*m*/*z*) 123 and 122, respectively, and dwell time 1 s.

2.4. Samples and sample preparation

Fresh and dry-cured pork samples were collected. One hundred and forty samples of fresh pork cuts were collected from three slaughterhouses within six months. All the samples were immediately analysed after the collecting in order to determine the natural niacin levels.

Table 1
Chromatographic conditions for the determination of niacin by a cation-exchange method

Column	*OmniPac PCX*–500 i.d. = 2 mm
Eluent	140 mM HCOOH – 15 mM NH_4COOH – 5% ACN
Flow rate	250 μl/min
Temperature	40 °C
Inj. loop	10 μL
Detection	UV (262 nm) – mass spectrometry
MS conditions	MSQ™ + ESI, 50 V, 350 °C, SIM as indicated

Fifty fresh cuts with different oxidative patterns from shoulder (red muscle – *Masseter*) and loin (white muscle – *Longissimus dorsi*) were taken from 50 carcasses and also 40 mixed white and red muscle cut samples, usually employed to prepare fresh sausage and salami, were collected.

Dry cured ham (12 months aged), fresh and dry-cured sausages (3 months aged) were randomly purchased from local markets.

In fresh meat and dry-cured meat, niacin was extracted by means of an acidic hydrolysis. An adequate amount of homogenised meat sample (5–10 g, containing 50–1000 μg of niacin) was weighed into a pyrex flask and 25 ml of 1 N hydrochloric acid and 5 ml of methanol were added. The solution was autoclaved at 121 °C for 30 min. After cooling, the excess proteins were precipitated with trichloroacetic acid and the solution was filled to 100 ml with distilled water. The solution was filtered first through filter paper and then through a cellulose acetate filter (0.45 μm) for HPLC analysis.

2.5. Experimental design

The response surface methodology (RSM) was used to study the simultaneous effects of two experimental factors (eluents concentration) on the chromatographic performance of niacin.

A 3-level-factorial design (Box & Behnken, 1960; Box, Hunter, & Hunter, 1978) was employed to study the relationship between the analytical conditions (independent variables) and the nicotinic acid resolution (response variable) and identify the optimum response region for the dependent variable. Three levels for each factor were chosen according to the experimental design, shown in Table 1, to describe first and second-order effects. By a 3^2 design the effects of the factors were studied with 14 runs including five replications of the central point. The order of the experiments was fully random.

Experimental design and statistical analysis were performed using Statgraphics Plus 5.1 for Windows (Statistical graphics Corp., Rockville, MD, USA)

3. Results and discussion

3.1. Optimization of nicotinic acid separation

The first step of the statistical approach to the analysis optimisation was to establish the criteria that will define the method quality (response variable) and the experimental factors that have a significant effect on the response variables. Because the major aim of this work was to achieve the best separation of nicotinic acid in the shortest analysis time, the key parameter chosen to describe the peak separation was resolution (R_s), calculated using the following equation:

$$R_s = \frac{2 \cdot t'_2 - t'_1}{w_1 + w_2},$$

where t'_i and w are peak retention time and width at the baseline, respectively.

The resolutions between the nicotinic acid and the first and the second interference peaks (peak n.1 and peak n.2 of the chromatogram in Fig. 1) are set as the two response variables to be studied.

In order to screen the appropriate experimental variables having an effect on the response variable and to determine the experimental domain, preliminary experiments were carried out. Therefore, the effect of the mobile phase concentration on the capacity factor (k') of nicotinic acid and interfering substances was studied in a one-at-a-time approach.

The plot of capacity factors logarithms of the analytes versus salt (Fig. 2) and acid concentrations (Fig. 3) showed a linear correlation. The best separation between the nicotinic acid peak and the first interfering peak occurred at lower salt concentration, while increasing the acid concentration improved the resolution between nicotinic acid and the second interfering peak. On the basis of these preliminary results, eluent concentrations were chosen as experimental factors having a significant effect ($P < 0.05$) on the response variable.

Since the organic modifier (acetonitrile) did not show a significant effect ($P < 0.05$) on the capacity factors of the analytes, its concentration was set to 5% (the lower concentration level as suggested by the column manufacturer).

A 3-level factorial design was employed to investigate the simultaneous effect of the formic acid (X_1) and ammonium formate (X_2) on the nicotinic acid resolution and to find the best eluent composition.

A multiple regression analysis of the data was used to describe the variables under study taking into account linear, quadratic and cross-product terms (Table 2) for each experimental variable. The significance of the equation parameters on nicotinic acid resolution was assessed by the F test.

Results evidenced that salt concentration (X_2) is a significant factor for both response variables model along with acid concentration (X_1^2) (as second-degree term) suggesting that the optimisation of nicotinic resolution requires a simultaneous effect of both the eluents.

The regression coefficient (r^2) for each response variable (resolution from the first and the second interferent peaks) were 0.898 and 0.841, respectively, indicating a good predictability of the models.

A three-dimensional plot of both modelled response surfaces and contour plot of the estimated surfaces are shown in Figs. 4 and 5. These graphical results (referred to nicotinic acid resolution from the first interferent

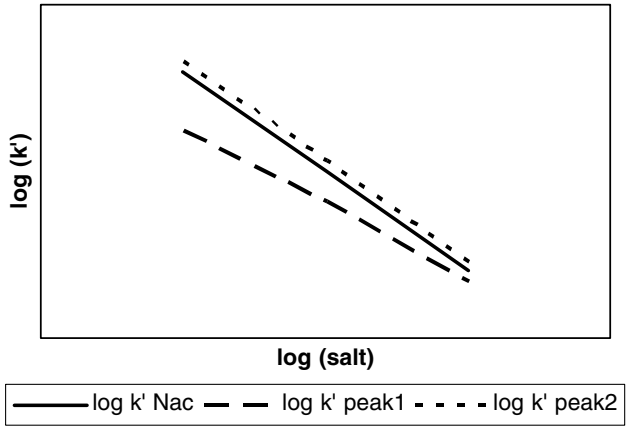

Fig. 2. Linear retention plot of capacity factors versus ammonium formate concentrations for the interferences (peak n.1 and peak n.2 showed in the Fig. 1) and nicotinic acid peaks (Nac).

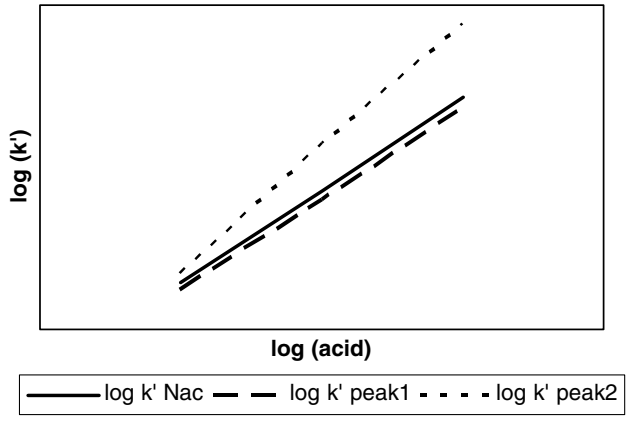

Fig. 3. Linear retention plot of capacity factors versus formic acid concentrations for the interferences (peak 1 and peak 2) and nicotinic acid peaks (Nac).

Table 2
Factors employed in the experimental design (3-level design)

Factors (mM)	Low level (−1)	Medium level (0)	High level (+1)
Formic acid	20	110	200
Ammonium formate	10	30	50

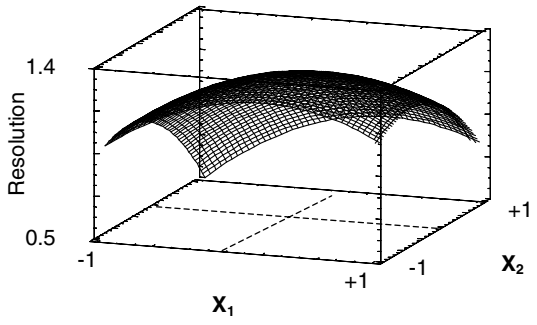

Fig. 4. Response surface of the nicotinic acid resolution as a function of formic acid [X_1] and ammonium formate [X_2] concentrations.

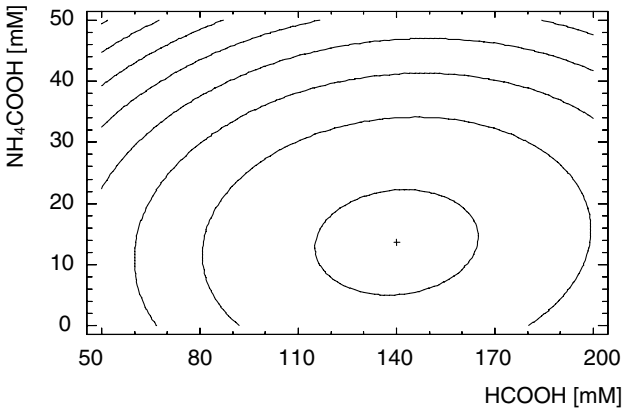

Fig. 5. Contour plot of estimated response surface of nicotinic acid resolution from The circled area in the central part design a resolution level best of one.

Table 3
Value and significance of the coefficients of the original multiple regression model

Parameter	Resolution from first interference peak		Resolution from second interference peak	
	Coefficient	Significance[a]	Coefficient	Significance[a]
Intercept parameter	0.5715	0.04	−0.1688	0.03
X_1	0.095	0.08	0.0429	0.25
X_2	0.051	0.02	−0.067	0.03
X_1^2	−0.000035	0.04	−0.0002	0.09
X_2^2	0.00002	0.14	0.0013	0.01
$X_1 X_2$	−0.00029	0.57	−0.0002	0.13
Regression model r^2	0.898		0.841	

[a] Significance level<0.05.

peak) are useful to evidence that the most pronounced effect is played by the salt concentration and also to identify the optimum region where the best peak resolution can be obtained. By comparing the optimum regions of both the response variables, a common maximum region was identified. On the basis of these results the mobile phase composition used in the following validation experiments was 140 mM formic acid and 15 mM ammonium formate in 5% acetonitrile.

The chromatogram reported in Fig. 6 was obtained under the optimised conditions and is related to a fresh sausage sample, where we observed the major interferences (see Fig. 1). In this chromatogram a good resolution between the nicotinic acid peak and the interfering peaks is evident. The presence of a peak at the same retention time of nicotinamide (Rt: 17.2 min.) suggested a confirmation analysis by mass spectrometry. From the chromatogram in Fig. 6 that shows the trace of SIM signals at 123 *m/z* [nicotinic acid + H]$^+$ and 122 *m/z* [nicotinamide + H]$^+$, it is evident the lack of nicotinamide in the sample. Quantitative determination using SIM signal at 123 *m/z* was in good agreement with results obtained by UV detection (0.98 correlation coefficient (see Table 3).

Table 4a
(a) Chromatographic performances of the optimised analytical method

	LOD (mg l^{-1})	Precision (CV)	
		Intra-day	Inter-day
Nicotinic acid	0.2	3.9	6.3

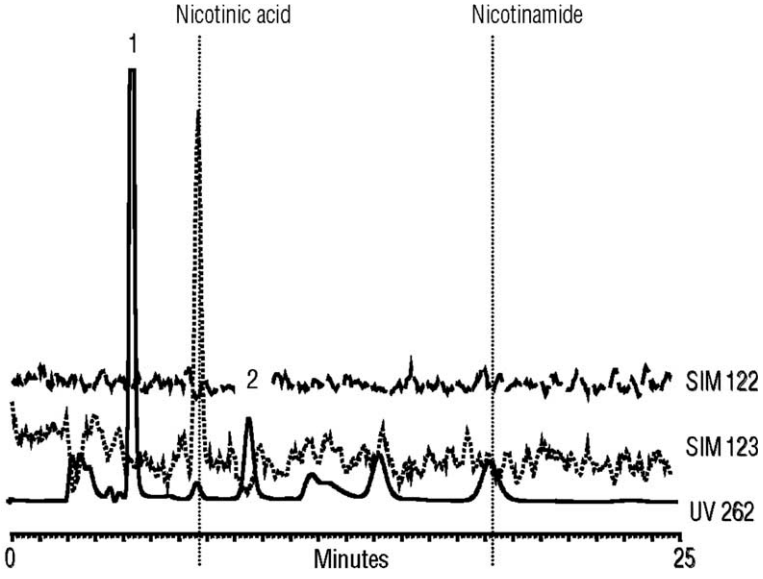

Fig. 6. Optimized chromatographic conditions as in Table 1 for a hydrolysed fresh pork sausage sample (d:10) and MS confirmation of effectiveness of hydrolysis procedure. Compared with the chromatogram in Fig. 1, the interference peaks 1 and 2 are clearly resolved from the nicotinic acid peak. There is no evidence of nicotinamide peak in the SIM chromatogram at 122 *m/z* [nicotinamide + H]$^+$ while the SIM signal at 123 *m/z* [nicotinic acid + H]$^+$ confirms the UV response of nicotinic acid.

Table 4b
(b) Repeatability and recoveries of nicotinic acid in fresh and dry-cured pork products

	Nicotinic acid		Fortification level			
			10 mg kg^{-1}		40 mg kg^{-1}	
	Average (mg kg^{-1})	CV	Recovery (%)	CV	Recovery (%)	CV
Fresh sausage ($N = 10$)	37	4.5	88	4.5	90	4.1
Dry-cured ham ($N = 10$)	56	3.9	93	4.1	95	3.8

Samples spiked with nicotinamide before the acidic hydrolysis.

3.2. Chromatographic performances

The chromatographic performances of the optimized method was further studied with respect to linearity, accuracy, precision and limit of detection and are reported in the Table 4a and 4b.

The linearity of nicotinic acid was checked over two orders of magnitude from 0.5 to 50 mg l^{-1} and the calibration curve for quantitative assay was calculated in a range between 0.5 and 10 mg l^{-1}. The limit of detection, as threefold the standard deviation of a blank sample, was 0.2 mg l^{-1}. To check the precision of the method the intra-day and inter-day repeatability were studied using one level of 5 mg l^{-1} of nicotinic acid ($N = 10$) (Table 4a).

The accuracy of the method was studied by spiked samples, with two levels of addition of nicotinamide, in order to include the hydrolysis procedure step, in fresh and dry cured pork products: for both matrices the recoveries were up to 88% (Table 4b).

The developed method was employed to characterize endogenous occurrence of niacin in fresh pork meat. Several fresh cuts with different oxidative patterns were chosen to identify the natural range of niacin: loin cuts were taken as an example of white muscle (*Longissimus dorsi*), shoulder (*Masseter*) as oxidative muscle and mixed cuts of fresh pork meat were also studied as frequently employed to prepare fresh and dry-cured sausages.

Samples of fresh pork meat ($N = 140$) were collected and analysed in order to determine the niacin content and to identify the natural niacin level range (Table 5): shoulder samples showed the highest content of niacin, while white muscles have the lowest level, in agreement with their oxidative pattern.

Table 5
Niacin in fresh and dry-cured pork samples

	N	Nicotinic acid (mg kg^{-1})
		Median (min–max)
Fresh pork samples		
Loin	50	28 (20–50)
Shoulder	50	45 (18–73)
Mixed cuts	40	33 (18–57)
Dry-cured pork samples		
Dry-cured ham	34	64 (29–170)
Dry-cured sausages	32	51 (24–117)

4. Concluding remarks

A robust, simple and selective chromatographic method based on cation-exchange separation and UV detection at 262 nm for the determination of niacin in meat and meat products has been developed. An improved resolution from interferences by using a 3-level factorial design was achieved and the use of IC-MS SIM signals at 123 *m/z* [nicotinic acid + H]$^+$ and 122 *m/z* [nicotinamide + H]$^+$ confirmed the effectiveness of the method selectivity and hydrolysis procedure.

The optimised method was demonstrated to be suitable to identify niacin occurrance in fresh cuts and dry cured pork products, but, in our opinion, it might be applied to any kind of meat.

Acknowledgement

The authors are particularly grateful to Dr. S. Polesello, CNR-IRSA, Brugherio (MI), for a critical review of the manuscript.

References

Ball, G. F. M. (1994). *Water-soluble vitamin assays in human nutrition*. London, UK: Chapman and Hall.

Balschukat, D., & Kress, E. (1990). Use of column-switching for the determination of niacinamide in compound feed. *Journal of Chromatography, 502*, 79.

Bertling, L., & Tietz, J. (1978). The use and determination of nicotinic acid in meat products. *Fleischwirtschaft, 58*, 621.

Box, G. E. P., Hunter, W. G., & Hunter, S. J. (1978). *Statistics for experimenters: An introduction to design, data analysis, and model building*. New York: Wiley.

Box, G. E. P., & Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics, 2*, 455.

Chase, G. W., Landen, W. O., Jr., Soliman, A. M., Jr., & Eitenmiller, R. R. (1993). Liquid chromatographic analysis of niacin in fortified food products. *Journal of AOAC International, 76*, 390.

Hamano, T., Mitsuhashi, Y., Aoki, N., Yamamoto, S., & Oji, Y. (1988). Simultaneous determination of niacin and niacinamide in meats by high-performance liquid chromatography. *Journal of Chromatography, 457*, 403.

Hirayama, S., & Maruyama, M. (1991). Determination of a small amount of niacin in foodstuffs by high-performance liquid chromatography. *Journal of Chromatography, 588*, 171.

Holasova, M., & Maskova, E. (2001). Niacin determination in meat and offals by HPLC and its comparison with microbiological assay. *Czechoslovak Journal of Food Science, 17*(5), 166.

http://www.europa.eu.int/comm/food//fs/sc/scf/out80j_en.pdf Opinion of the Scientific Committee on Food on The tolerable upper intake levels of nicotinic acid and nicotinamide (Niacin).

http://www.nutrition.org/nutinfo/content/niac.shtml.

Kendrick, J. L., & Watts, B. M. (1969). Nicotinamide and nicotinic acid in color preservation of fresh meat. *Journal of Food Science, 34*, 292.

Krishnan, P. G., Mahmud, I., & Matthees, D. P. (1999). Postcolumn fluorimetric HPLC procedures for determination of niacin content of cereals. *Cereal Chemistry, 76*(4), 512.

LaCroix, D. E., & Wolf, W. R. (2001). Determination of niacin in infant formula by solid-phase extraction and anion-exchange liquid chromatography. *Journal of AOAC International, 84*(3), 789.

Lahély, S., Bergaentzlè, M., & Hasselmann, C. (1999). Fluorimetric determination of niacin in food by high-performance liquid chromatography with post column derivatization. *Food Chemistry, 65*, 129.

Polesello, A., & Rizzolo, A. (1986). Applications of HPLC to the determination of water-soluble vitamins in foods (a review 1981-1985). *Journal of Microbial Analysis, 2*(3), 153–187.

Rizzolo, A., & Polesello, S. (1992). Chromatographic determination of vitamins in food. *Journal of Chromatography, 624*, 103.

Rizzolo, A., & Polesello, S. (1998). Chapter16: Vitamins. In Z. Deyl (Ed.), *Advanced chromatographic and electromigration methods in Biosciences* (pp. 652–730). Amsterdam: Elsevier.

Takatsuki, K., Suzuki, S., Sato, M., Sakai, K., & Ushizawa, I. (1987). Liquid chromatographic determination of free and added niacin and niacinamide in beef and pork. *Journal of AOAC International, 70*(4), 698.

Tyler, T. A., & Genzale, J. A. (1990). Liquid chromatographic determination of total niacin in beef, semolina and cottage cheese. *Journal of AOAC International, 73*(3), 467.

Vidal-Valverde, C., & Reche, A. (1991). Determination of available niacin in legumes and meat by high-performance liquid chromatography. *Journal of Agricultural Food Chemistry, 39*, 116.

Ward, C. M., & Trenerry, V. C. (1997). The determination of niacin in cereals, meat and selected foods by capillary electrophoresis and high-performance liquid chromatography. *Food Chemistry, 60*, 667.