DETERMINATION OF 4-HYDROXY-2-NONENAL IN PORK PRODUCTS

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Background

4-hydroxy-2-nonenal (4-HNE) is an unsaturated and hydroxylated aldehyde which represents one of the major products of membrane lipid oxidation. 4-HNE derives in particular from ω-6-polyunsaturated fatty acids such as arachidonic and linoleic acid. Due to its high reactivity 4-HNE is implicated as a mediator of diverse biological effects but it is considered to be very toxic to mammalian cells at levels higher than physiological ones. Concentrations of 4-HNE below 0.1 μM occur in many tissues, as well as in serum, and the effect observed at such levels may therefore be of physiological significance. 4-HNE at concentrations higher than 100 μM is a substrate for glutathione conjugation through SH-group resulting in depletion of cellular glutathione (Esterbauer et al., 1991) and it has the ability to react with lysine residues of human low density lipoproteins forming oxidised-LDLs involved in the formation of atherosclerotic plaques (Requena et al., 1997); genotoxic potential of 4-HNE is supported by the formation of adducts with DNA by amino groups serving as nucleophiles (Witz, 1989).
Despite the link between lipid oxidation products and some human pathologies is mostly not a direct cause-effect relationship, cardiovascular pathologies are among the diseases linked to oxidative stress. 4-HNE in particular has been proposed as an important marker of radical induced lipid peroxidation and abundant clinical data are available in literature. Besides nutritional consequnces, organoleptic aspects such as meat colour can be affected by unsaturated aldehydes. 4-HNE in particular, can alter myoglobin stability through an increase of oxymyoglobin oxidation and a decrease of the ability of metmyoglobin to be reduced by enzymes (Lynch and Faustman, 2000).
Only a few reports deal with the levels of 4-HNE in meat and meat products. According to Sakai et al. (1995) the range of values of 4-HNE is 2-23 mg/kg and 0.2-24 mg/kg in beef and pork meat respectively. Lower content has been detected by Lang et al. (1985) in fried or roasted pork cutlets (0.2 mg/kg) and in roasted chicken (0.1 mg/kg). The lack of data is probably due to the absence of a specific analytical method for the determination of 4-HNE in meat tissues.
Immunochemical reactions based on monoclonal antibodies specific for 4-HNE are the analytical procedures generally employed for the detection of 4-HNE-protein adducts in clinical field. The detection of free 4-HNE in biological fluids and lipid oxidation model systems is performed after derivatization by gas chromatography-mass spectrometry (GC-MS) or high pressure liquid chromatography (HPLC) coupled with UV, electrochemical or fluorescence detector. Recently Gioacchini et al. (2000) have proposed HPLC coupled with triple quadrupole mass spectrometer detector (HPLC-MS/MS) as a specific and sensitive analytical method for the determination of free 4-HNE at cellular level. The methods validated for biological fluids or lipid model systems were not found to be sufficiently repeatable and efficient in terms of recovery when applied to meat products.

Objectives

The present study was aimed at developing a reliable method based on solid phase extraction (SPE) and HPLC-MS/MS for the identification and measurement of free 4-HNE in pork products of various types. The SPE conditions have been optimised in order to achieve highest recovery and chromatographic / mass spectrometry parameters have been tuned to reach highest levels of selectivity and sensitivity.

Methods

Sampling - Thirty-six pork products of different brands were purchased from local supermarkets and precisely : eight Milano sausages, four Cacciatore sausages, four zamponi, four smoked sausages and four packs of frankfurters bought as whole pieces; four different samples of mortadella, Parma ham and cooked ham were purchased as sliced products in 100 g protective atmosphere packs. Whole products were kept at room temperature and sliced products were stored refrigerated.
Comminuted meat tissues (10 g) were homogenised in 20 ml of water. The homogenate was centrifuged for 10 min. at 5000rpm and 4°C and the residue was further homogenized after filtration of the upper liquid phase. The joint supernatant phases were submitted to solid phase extraction by an ODS stationary phase column. Residual lipid substances were eluted by 15 ml of petroleum ether whereas 4-HNE was eluted with 2 ml of methanol. 50 μl of the extract were injected in HPLC system (Spectra Physics P2000) equipped with reverse phase column LiChrospher 100 RP18 (250 x 4 mm, 5 μm) and a triple quadrupole mass spectrometer (Applied Biosystem, API 365) for the identification and quantification. Methanol acidified with 0.1% of formic acid was used as mobile phase at 0.8 ml/min. constant flow. Ionization of 4-HNE was obtained by Turbo-Ionspray in the positive ion mode. MS spectra of 4-HNE, obtained by direct infusion of 1mg/l methanol solution in the *m/z* 100-200 mass range, showed two ions at *m/z* 157 and *m/z* 171 (base peak). The first was the molecular ion $[M+H]^+$ and the secon could be considered as the acetal of 4-HNE due to the reaction between 4-HNE and methanol followed by loss of a water molecule. The collisional spectra of *m/z* 171 ion were obtained in the range *m/z* 50-180. MS/MS acquisition was performed in the mass reaction monitoring (MRM) mode by monitoring the reaction m/z 171 → 69 characteristic of 4-HNE.
Preparation of calibration standards - In order to evaluate the linear dynamic range, calibration standards at 0.1-0.5-1-5-10 mg/kg were prepared by spiking Milano sausages with appropriate volumes of 4-HNE standard solutions. Three samples for each concentration were submitted to solid phase extraction and HPLC-MS/MS analysis. Calibration curve was constructed by plotting the concentration against peak area (n=15) using unweighted linear regression model $y=a+bx$. Repeatability was determined at two concentration levels (0.1 and 10 mg/kg) from six repeated experiments (n=6). Intra-day precision was determined at two concentration levels (0.1 and 10 mg/kg) from six repeated experiments performed two times during a working day (n=12). Inter-day variability was evaluated on three consecutive working days from the analysis of six repeated experiments (n=18) at two concentration levels (0.1 and 10 mg/kg).
Percent of recovery of 4-HNE was calculated at two concentration levels (1 and 10 mg/l) from six pure standard methanolic solutions and at two concentration levels (0.1 and 1 mg/kg) from six spiked samples of Milano sausage.
The limit of detection (LOD) of 4-HNE, defined as the amount injected giving a signal-to-noise ratio of 3, was determined by direct injection of standard solutions and by injection of spiked Milano sausage after SPE cleanup.

Results and discussion

Validation of the method – The linearity was explored using spiked sausage samples in the concentration range 0.1-10 mg/kg. The coefficients of calibration curve are reported in Table 1. A good linearity of the method was observed with correlation coefficient r = 0.982. The recovery of 4-HNE pure standard was complete (98-110%) both at 1 and 10 mg/L concentration. Percent of recovery of 4-HNE in spiked samples was 63.0 ± 7.2 at 0.1 mg/kg and 59.7 ± 3.5 at 1 mg/kg concentration. Much lower recovery, around 10-12%, was obtained

when a mixture water/methanol (80/20, v/v) was used to homogenize the sample and to elute 4-HNE as suggested by Lang et al. (1985). Attempts to perform the extraction of 4-HNE by acetonitrile failed due to the formation of a massive precipitate.
The limit of detection of 4-HNE standard solution without any sample enrichment was 0.005 mg/l, in agreement with Gioacchini et al. (2000) who proposed triple quadrupole mass spectrometry for the determination of 4-HNE in human T leukemia extracts and found a LOD of 0.002 mg/l. The LOD of 4-HNE in spiked Milano sausage sample was 0.043 mg/kg; it must be emphasized that this result was not corrected for recovery efficiency (Table 2).
Repeatability, intra-day and inter-day precision were calculated as relative standard deviation on repetitive experiments of spiked sausage samples at two concentration levels and are reported in Table 3.

Table 1. Coefficients of linear regression model $y=a+bx$ (n=15)

Range (mg/kg)	*a*	*b*	r
0.1 – 10	6453 ± 1947*	8758 ± 424*	0.982

* mean ± standard error

Table 2. Limit of detection (LOD)

4-HNE	LOD
Pure standard	0.005 mg/l
Spiked sample	0.043 mg/kg

Table 3. Repeatability, intra- and inter-day precision calculated as relative standard deviation on repetitive experiments

Compound	Concentration (mg/kg)	Repeatability (n=6) (%)	Intra-day precision (n=12) (%)	Inter-day precision (n=18) (%)
4-HNE	0.1	6.9	8.7	9.1
	10	7.3	5.8	9.4

Table 4. 4-HNE content in pork products

Sample	Concentration (mg/kg)
Milano sausage (n=8)	n.d.*
Cacciatore sausage (n=4)	n.d.
Smoked sausage 1	n.d.
Smoked sausage 2	n.d.
Smoked sausage 3	0.41 ± 0.04
Smoked sausage 4	0.28 ± 0.14
Zampone 1	n.d.
Zampone 2	0.34 ± 0.09
Zampone 3	0.13 ± 0.03
Zampone 4	0.15 ± 0.06
Parma ham (n=4)	n.d.
Cooked ham (n=4)	n.d.
Frankfurter 1	0.71 ± 0.14
Frankfurter 2	0.62 ± 0.05
Frankfurter 3	0.27 ± 0.05
Frankfurter 4	0.46 ± 0.04
Mortadella 1	0.06 ± 0.01
Mortadella 2	0.16 ± 0.04
Mortadella 3	0.32 ± 0.05
Mortadella 4	0.08 ± 0.02

*n.d. = lower than LOD

Application - The validated method was applied to preliminary investigation of 4-HNE content in pork products chosen among those considered representative and highly consumed in Italy. 4-HNE content in most of the products tested in the present study was under the limit of detection. The level of 4-HNE was constantly lower than 0.043 mg/kg in Milano and Cacciatore sausages, in two smoked sausages, in one zampone, in Parma hams and in cooked hams. The level of 4-HNE in the other products was between 0.06 and 0.71 mg/kg. Among the raw products, only two North Europe smoked sausages had values higher than the limit of detection and one of them reached 0.41 mg/kg. Values were generally above the limit of detection in cooked products with the exception of cooked hams and one zampone. Mortadella and zampone, cooked at higher temperatures than cooked hams (not smoked) had intermediate levels whereas the highest concentrations, up to 0.71 mg/kg, were observed in frankfurters, smoked cooked products. The values, expressed on a molar basis, varied therefore from 0.38 to 4.6 µM approximately. In some cases, therefore, 4-HNE concentrations were just above the levels considered physiological for many tissues but what is not known, at the moment, is the degree of absorption of 4-HNE from the diet and therefore the real risk for the human health cannot be realised

Conclusions

The method developed for the detection and quantification of 4-hydroxy-2-nonenal in pork products has proved to be sensitive and reliable.
The limit of detection was 0.043 mg/kg and the recovery was about 60% depending on the concentration.
Good linearity of the method was observed and repeatability, inter-day and intra-day precision expressed as relative standard deviation were lower than 10%.
4-HNE concentration found in pork products were in the range 0.06±0.01 and 0.71±0.14 mg/kg. The content of 4-HNE in Milano sausage, Cacciatore sausage, Parma ham and cooked ham was constantly below the limit of detection whereas two Northern type smoked, three zampone, mortadella and frankfurters showed highest values. The values normally found, probably, are not a real risk for human health.

Pertinent literature

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