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Solid Phase Extraction and Liquid Chromatography−Tandem Mass Spectrometry for the Evaluation of 4-Hydroxy-2-nonenal in Pork Products

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This research was aimed at setting up an analytical method for the determination in pork products of 4-hydroxy-2-nonenal (4-HNE), an aldehyde produced from the oxidation of ω-6-polyunsaturated fatty acids. Such a compound mediates various biological effects, but it is considered to be very toxic to mammalian cells at levels higher than physiological ones. The methods used for the determination of 4-HNE in biological fluids, such as blood, were found to be unsuitable for meat samples because both the repeatability and the recovery in spiked samples were unsatisfactory. A new method, based on solid phase extraction and HPLC-MS/MS, was therefore developed and validated. The limit of detection of 4-HNE in spiked samples was 0.043 mg/kg, and the recovery was ~60% depending on the concentration. Good linearity was observed in the range of 0.1−10 mg/kg, and repeatability and interday and intraday precision expressed as relative standard deviation were <10%. The method has been successfully applied to the determination of the aldehyde in samples of various pork products. 4-HNE was present in some products, especially the smoked and/or cooked ones, at levels that might not be a real risk for human health.

KEYWORDS: 4-Hydroxy-2-nonenal; lipid oxidation; pork products; safety

INTRODUCTION

The aldehydes are carbonyl compounds that play a pivotal role in the aroma of some meat products. Methyl-branched aldehydes, produced by microbial catabolism or by Strecker degradation of amino acids (*1*, *2*), have been related to typical dry sausage aroma (*3*) and to the aged and cured flavor of Parma and Iberian hams, respectively (*4*, *5*). Straight-chain aldehydes, originated from oxidation of polyunsaturated fatty acids of cell membranes, make up the major class of volatiles, after sulfur compounds and terpenes produced from spices, in the traditional Italian fermented sausages, (*6*, *7*) and in French dry-cured ham (*8*).

However, hydroxylated aldehydes act also as mediators of various biological effects and, depending on their concentration, can be a risk factor for human health. 4-Hydroxy-2-nonenal (4-HNE) is a typical example of a hydroxylated aldehyde with a chain of nine carbon atoms, a double bond, and a hydroxyl group on carbons 2 and 4, respectively (**Figure 1**). The aldehyde is one of the major products of membrane lipid oxidation and derives, in particular, from ω-6-polyunsaturated fatty acids such as arachidonic and linoleic acid. Concentrations below 0.1 μM of 4-HNE occur as basal level in many tissues, as well as in serum, and the effects observed at such levels may therefore be of physiological significance. Among known physiological actions at such concentrations there is the stimulation of oriented migration of neutrophilis (*9*), the modulation of adenylate cyclase activity, and the stimulation of guanylate cyclase (*10*). 4-HNE concentrations in the range of 1−20 μM can inhibit DNA and protein synthesis, and a growing number of studies suggest that such effects can be produced in response to oxidative stress (*11*−*13*). At such concentrations the cytotoxic effects of 4-HNE are selective, and significant differences exist among different cell types. Isolated hepatocytes are much more resistant and not lethally damaged compared to endothelial cells of human umbilical vein, and their higher resistance may in part be due to a higher capacity to metabolize and detoxify the aldehyde (*14*). Concentrations of 100 μM and higher cause acute and unspecific cytotoxic effects leading in most cases to rapid cell death. Many basic catabolic and anabolic cell functions are

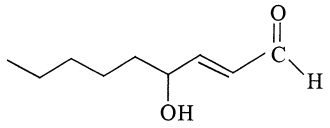

Figure 1. Structure of *trans*-4-hydroxy-2-nonenal (4-HNE).

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partially or fully inhibited by such high concentrations. 4-HNE at concentrations higher than 100 μM is a substrate for glutathione conjugation through the SH group, resulting in the depletion of cellular glutathione (*14*), and it has the ability to react with lysine residues of human low-density lipoprotein (LDL) forming oxidized LDLs, compounds considered to be important for the development of atherosclerotic plaques (*15*). The genotoxic potential of 4-HNE is supported by the formation of adducts with DNA by amino groups serving as nucleophiles (*16*).

Chronic cardiovascular diseases have been associated with oxidative stress. The link between lipid oxidation products and some human pathologies, however, is mostly not a direct cause−effect relationship. 4-HNE in particular has been proposed as an important marker of radical-induced lipid peroxidation, and abundant clinical data are available in the literature. A fluorescent derivative, from the reaction of 4-HNE with lysine residues, has been found in oxidized plasma LDLs of atherosclerotic patients (*17*). The same derivative has been reported to accumulate in atherosclerotic lesions of the human aorta (*18*). Romero et al. (*19*), moreover, have pointed out and discussed the possible importance of 4-HNE accumulation in the brain of patients with Alzheimer's disease.

The aldehydic products of lipid oxidation in food could have therefore nutritional implications because, together with cholesterol oxides, they can be absorbed from the human intestinal tract and found in chylomicrons and other lipoproteins (*20*, *21*). Besides nutritional consequences, quality aspects such as meat color can be affected by unsaturated aldehydes. 4-HNE in particular can negatively affect myoglobin stability by increasing oxymyoglobin oxidation and decreasing enzymic reduction of metmyoglobin (*22*).

Immunochemical reactions based on monoclonal antibodies specific for 4-HNE are generally employed to detect 4-HNE-protein adducts in the clinical field (*23*−*25*). The detection of free 4-HNE in biological fluids and lipid oxidation model systems is performed by derivatization followed by the measurement of derivatized compounds by gas chromatography−mass spectrometry (GC-MS) (*26*−*28*) or high-performance liquid chromatography (HPLC) through electrochemical (*29*), ultraviolet (UV) (*30*), or laser fluorescent detection (*31*, *32*). Electrospray mass spectrometry (MS) and triple-quadrupole mass spectrometry (MS/MS) have been recently proposed as analytical methods for the determination of 4-HNE in tumor cellular extracts (*33*, *34*).

Only a few papers deal with the levels of 4-HNE in meat and meat products, and the lack of data is probably due to the absence of a specific analytical procedure for muscle tissues. The methods validated for biological fluids or lipid oxidation model systems were not found to be sufficiently repeatable when applied to meat products. It was deemed necessary, therefore, to develop a specific and sensitive method based on solid phase extraction and HPLC-MS/MS for the identification and measurement of 4-HNE in meat products. The choice of the products to be tested has fallen on a few pork products considered to be representative for oxidative risks.

MATERIALS AND METHODS

Chemicals. Solvents used for solid phase extraction and chromatographic analysis were of HPLC grade (LabScan, Dublin, Ireland). *trans*-4-Hydroxy-2-nonenal was obtained from Oxis (Portland, OR). Standard stock solutions (1 g/L) were prepared in methanol. The solutions were stable for at least 1 month when stored in the dark at −70 °C. Analytical grade formic acid and anhydrous sodium sulfate were supplied by Carlo Erba Reagenti (Rodano, Italy).

Sampling. Thirty-six commercial pork products were randomly chosen among different brands available in local supermarkets: eight Milano sausages, four Cacciatore sausages, four zampone, 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 to mimic the commercial conditions of storage until the analysis was performed (maximum 2 days). Milano and Cacciatore sausages were examples of Mediterranean dried fermented sausages. Both are traditional Italian pure pork sausages differing mainly in size and maturation period, respectively higher and longer for Milano sausages. Smoke is not used, and the final pH is ∼5.5. Smoked sausages belonged to a northern European type of pork and beef sausages, characterized by quick fermentation, a short drying and ripening period, and final pH values <5. Zampone, mortadella, and frankfurters represented minced cooked pork products: zampone is a mixture of lean, fat, and pork rind stuffed in the skin of the forelegs and sterilized in aluminum pouches; mortadella is a big size minced and cooked emulsion-type product with the addition of small lard cubes from streaky bacon cooked to ∼80 °C core temperature; frankfurters are a well-known smoked and cooked small size minced emulsion type product. Hams were examples of whole muscle products: Parma ham is dry salted and matured for a minimum of 12 months, whereas cooked ham is cooked to an internal temperature of ∼70 °C.

Solid Phase Extraction (SPE). Comminuted meat tissues (10 g) were homogenized in 20 mL of distilled water by an Ultra-Turrax T25 homogenizer (Staufen, Germany) at medium speed for 1 min. The homogenate was centrifuged for 10 min at 5000 rpm and 4 °C. The upper liquid phase was filtered by a filter paper, and the residue was further homogenized in 20 mL of distilled water in the conditions previously described. After a second centrifugation at 5000 rpm and 4 °C for 10 min, the joint upper filtered phases (∼35 mL) were loaded on an SPE C18 end-capped cartridge (Isolute, 500 mg, 3 mL volume, International Sorbent Technology, Mid Glamorgan, U.K.) previously equilibrated with 3 mL of methanol and 3 mL of distilled water. Residual lipid substances were eluted by 15 mL of petroleum ether. Elution of 4-HNE was obtained by 2 mL of methanol. The equilibration of the cartridge and the elution of the different solvents were performed by gravity. Residual water was eliminated by anhydrous sodium sulfate. After filtration and evaporation to dryness by a steam of nitrogen, the sample was dissolved in 1 mL of methanol acidified by 0.1% formic acid and filtered through a 0.45 μm PTFE filter. Fifty microliters of the sample was injected in the HPLC-MS/MS system. Each analysis was run in duplicate.

Liquid Chromatography—Mass Spectrometry. HPLC was carried out using a P2000 series binary pump (Spectra Physics, Milan, Italy) equipped with an AS3000 autosampler (Thermo Finnigan, San Jose, CA) provided with a 50 μL loop. The analyte was separated on a LiChrospher 100 RP18 column (250 mm × 4 mm i.d., 5μm, Merck, Darmstadt, Germany) by using methanol acidified by 0.1% formic acid as mobile phase at 0.8 mL/min. constant flow. The column effluent was connected to the Peek transfer line (1 m × 0.25 mm) of the mass spectrometer interface and subjected to a 70:30 split ratio. MS/MS analyses were carried out on an API 365 triple-quadrupole mass spectrometer (Applid Biosystems, Foster City, CA) equipped with a TurboIonSpray interface for pneumatically assisted electrospray. Ionization was obtained in the positive ion mode. The optimization of the interface parameters was performed by infusing 4-HNE standard solutions (1 mg/L methanol acidified by 0.1% formic acid). Electrospray conditions were as follows: nitrogen curtain gas, 14; air nebulizer gas, 9; ionspray voltage, 5500 V; temperature of nitrogen turbo gas, 300 °C; declustering potential, 20 V; focusing potential, 320 V. Total ion single-quadrupole mass spectra were acquired in the *m*/*z* 100−200 mass range, 0.60 s/scan. Product ion mass spectra of the *m*/*z* 171 ion were obtained in the range *m*/*z* 50−180, 0.60 s/scan. The fragmentation conditions were optimized by varying the nitrogen collision gas between 1 and 12 and the collision energy between 5 and 50 eV. MS/MS acquisition was performed in the mass reaction monitoring (MRM) mode by monitoring the reaction *m*/*z* 171 → 69 characteristic of 4-HNE.

The collision energy was 18 eV and the scan rate 0.60 s. The channel electron multiplier was set at 2300 V.

Preparation of Calibration Standards. 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 standard solutions containing 4-HNE to achieve the required concentration. Three samples for each concentration were submitted to SPE and to HPLC-MS/MS analysis. The integration of HPLC-MS/MS peaks obtained by monitoring the reaction *m/z* 171 → 69 was performed by the Analyst processing software. The calibration curve was constructed by plotting the concentration against peak area using an unweighted linear regression model ($n = 15$).

Repeatability was determined at two concentration levels (0.1 and 10 mg/kg) from six repeated experiments. Intraday precision was determined at two concentration levels (0.1 and 10 mg/kg) from six repeated experiments performed two times during a working day ($n = 12$). Interday 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).

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.

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.

RESULTS AND DISCUSSION

4-HNE Extraction. One of the most critical steps in the determination of 4-HNE in meat tissues is the extraction of the aldehyde from the matrix. Meat products, compared to biological fluids or lipid oxidation model systems, are difficult to handle due to their lower homogeneity and the frequently high amount of adipose tissue. The probable low concentrations of 4-HNE and the presence of many other substances require an efficient sample cleanup as well as a concentration step. The SPE was adopted with that aim. The experimental conditions were optimized by employing various solvents or their mixtures. The best results have been obtained by using water to homogenize and load the sample on the SPE cartridge and methanol to elute 4-HNE. In such working conditions the recovery of 4-HNE pure standard was complete (98−110%) at both 1 and 10 mg/L concentrations. Percentages of recovery of 4-HNE in spiked samples were 63.0 ± 7.2 at 0.1 mg/kg concentration and 59.7 ± 3.5 at 1 mg/kg concentration. A much lower recovery, ∼10−12%, was obtained when a mixture of water/methanol (80:20, v/v) was used to homogenize the sample and to elute 4-HNE as suggested by Lang et al. (*30*). Attempts to perform the extraction of 4-HNE by acetonitrile failed due to the formation of a massive precipitate. Such a precipitate was also observed when methanol was used for the extraction and the sample was resuspended in acetonitrile after methanol evaporation.

The derivatization is a classical step in the analysis of carbonyl compounds. The aim of this reaction is to stabilize the carbonyl group and to enhance the sensitivity of detection. A commonly used derivatizing agent of 4-HNE is 2,4-dinitrophenylhydrazine, which converts the aldehyde into its 2,4-dinitrophenylhydrazone (*29*). This procedure, adopted after distillation or solvent extraction of 4-HNE from meat samples, suffered from lack of repeatability, probably due to the artificial formation of 4-HNE from precursors catalyzed by the strongly acidic pH of the derivatization medium. Mild reaction conditions have been described for different derivatization methods for both gas and liquid chromatography analysis. Nuijens et al. (*31*) and Liu et al. (*32*) proposed new fluorescence labeling reagents for laser fluorescence detection of aldehydes. Other authors employed methods based on the determination of pentafluorobenzyloxime (*27*) or trimethylsilyl derivatives of 4-HNE (*28*) for human plasma. The procedures, though, are cumbersome and time-consuming and, some of them, are not specific for 4-HNE. In the present study, the high specificity and sensitivity of MS/MS analysis avoided the need of derivatization.

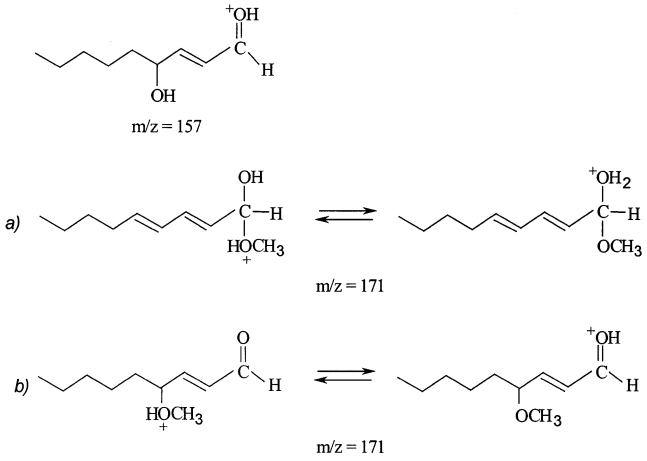

Figure 2. Structure of molecular ion (*m/z* 157) and proposed structures of *m/z* 171 ion (base peak).

Table 1. Calibration Graph for HPLC-MS/MS Analysis of 4-HNE[a]

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

[a] Calibration fitting: $y = a + bx$ ($n = 15$). Values given as mean ± standard error.

Validation of HPLC-MS/MS Method. Positive ionization was preferred over negative ionization because the latter produced signals of lower intensity. Positive ion MS spectra of 4-HNE obtained by direct infusion of 1 mg/L methanol solution showed two ions at *m/z* 157 and 171 (base peak). The first was the molecular ion $[M + H]^+$ and the second could be considered as the acetal of 4-HNE due to the reaction between 4-HNE and methanol, followed by further loss of a water molecule (**Figure 2**). To monitor only one ion, this reaction was favored by adding 0.1% formic acid to the methanol solution as proposed by Gioacchini et al. (*34*). The MS spectra of the acidified solution showed the complete disappearance of the $[M + H]^+$ ion and the presence of only the *m/z* 171 ion. The collisional spectra of the *m/z* 171 ion presented stable ions at *m/z* 139, 69, and 71. In agreement with Gioacchini et al. (*33*), the loss of methanol from the *m/z* 171 ion originated *m/z* 139; the cleavage of the covalent bond between carbons 4 and 5 of the latter gave rise to the *m/z* 71 and 69 ions. Due to the high relative abundance of the *m/z* 69 ion (2×10^7 cps) this ion was chosen for 4-HNE quantification.

The applicability of the HPLC-MS/MS method was evaluated after a careful study of the following parameters: linearity, precision, and limit of detection. The linearity was explored using spiked sausage samples in the concentration range of 0.1−10 mg/kg. Regression data are reported in **Table 1**. Experimental data fitted the linear model $y = a + bx$. A good linearity was observed as calibration showed a 2 orders of magnitude linear behavior with a correlation coefficient *r* of 0.982. The repeatability and intra- and interday precision were determined at two concentration levels, and the results expressed as relative

Table 2. Repeatability and Intra- and Interday Precision Calculated as RSD (Percent) at Two Concentration Levels of 4-HNE

concn (mg/kg)	repeatability ($n = 6$)	intraday precision ($n = 12$)	interday precision ($n = 18$)
0.1	6.9	8.7	9.1
10	7.3	5.8	9.4

Table 3. 4-HNE Content in Meat Products (Mean ± Standard Deviation)

sample	concn (mg/kg)
dry fermented sausages	
Milano sausage ($n = 8$)	nd[a]
Cacciatore sausage ($n = 4$)	nd
quick dry-fermented sausages	
smoked sausage 1	nd
smoked sausage 2	nd
smoked sausage 3	0.41 ± 0.04
smoked sausage 4	0.28 ± 0.14
dry salted hams	
Parma ham ($n = 4$)	nd
cooked hams	
cooked ham ($n = 4$)	nd
minced cooked pork products	
zampone 1	nd
zampone 2	0.34 ± 0.09
zampone 3	0.13 ± 0.03
zampone 4	0.15 ± 0.06
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

[a] nd, lower than LOD.

standard deviation (RSD) are summarized in **Table 2**. The data, ranging from 5.8 to 9.4%, prove that the repeatability of the method was satisfactory.

The LOD of the 4-HNE standard solution without any sample enrichment was 0.005 mg/L (0.03 μM), in agreement with Gioacchini et al. (*34*), 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 (0.01 μM). These values are in the range of concentration generally found as basal physiological level in serum and biological fluids. 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.

Field Tests. The validated method was applied to the preliminary investigation of 4-HNE content in pork products chosen among those considered to be representative and widely consumed.

Lipid oxidation of some traditional pork products has been previously investigated by measuring TBARS and cholesterol oxides content (*35*−*37*), but no data were available on 4-HNE content. A few studies have been dedicated to fresh and cooked meats. According to Sakai et al. (*38*) 4-HNE values vary between 2 and 23 mg/kg in beef and between 0.2 and 24 mg/kg in pork. Lower contents have been reported by Lang et al. (*30*) in fried or roasted pork cutlets (0.2 mg/kg) and in roasted chicken (0.1 mg/kg).

4-HNE contents in most of the products tested in the present study were under the LOD (**Table 3**). Milano and Cacciatore sausages, Parma hams, cooked hams, two northern European sausages, and one zampone had 4-HNE contents constantly <0.043 mg/kg, the LOD. The level of 4-HNE in the other products was between 0.06 and 0.71 mg/kg. It is interesting to note that, among the raw products, only two northerm European fermented sausages (smoked) had values higher than the LOD and one of them reached 0.41 mg/kg. Values were generally above the LOD in cooked products with the exception of cooked ham 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 to be physiological for many tissues. What is not known at the moment is the degree of absorption of 4-HNE from the diet, and therefore the real risk for human health cannot be realized.

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 ∼60% depending on the concentration. Good linearity of the method was observed, and repeatability and inter- and intraday precision expressed as relative standard deviation were <10%.

The concentrations found in pork products were in the ranges 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 LOD, whereas two northern European type smoked sausages, three zampone, mortadella, and frankfurters showed higher values. The values normally found, probably, are not a real risk for human health. However, given the high biological reactivity of 4-HNE, particularly with sulfhydryl groups of proteins, it would be advisable to conduct further investigations on the presence of 4-HNE−protein adducts in other meat products in relation to production technology and storage/marketing systems.

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