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Simultaneous HPLC Analysis of α-Tocopherol and Cholesterol in Fresh Pig Meat

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A method has been developed for simultaneously determining α-tocopherol and cholesterol in fresh pig meat by HPLC. It allows a reduction in the number of analyses and brings savings in time and materials. The unsaponifiable fraction is extracted following the modified method of Liu et al. (Liu, Q.; Scheller, K. K.; Schaefer, D. M. Technical note: A simplified procedure for vitamin E determination in beef muscle. *J. Anim. Sci.* **1996**, *74*, 2406−2410). The modifications introduced are the use of nitrogen atmosphere during the extraction, the addition of an antioxidant in the organic extraction phase, and the use of α-tocopherol itself as an internal standard. There is then a chromatographic analysis which allows the separation of the two compounds in question. To identify and quantify, two different detectors are used in series: the first is a fluorescence detector (α-tocopherol), and the second is a light-scattering detector (cholesterol). The technique shows sufficient sensitivity to determine the normal levels of α-tocopherol and cholesterol in meat, with recovery percentages of 78% and 97%, respectively. The average amount of α-tocopherol and cholesterol in samples from pig *Longissimus dorsi* muscle analyzed using this method is 1.8 and 620 mg/kg of fresh meat, respectively.

KEYWORDS: Meat; α-tocopherol; cholesterol; HPLC; fluorescence; light scattering

INTRODUCTION

Lipid oxidation is considered to be one of the main causes of deterioration in the sensorial and hygienic quality of meat (*1*). The supplementation of animal diet with α-tocopherol acetate reduces lipid oxidation and drip losses and improves the color of the meat (*2*, *3*). When administered as part of the diet, it increases the levels of α-tocopherol in the meat tissue, making its antioxidant effect more efficient than with exogenous application of the same substance (*4*). To evaluate the usefulness of this supplement, it is as important to analyze its effect on the quality of the meat as it is to learn the exact form of the α-tocopherol concentration in the muscle tissue. For this reason, in recent years various methods of analysis have been developed (*5*−*8*) among others, applicable to different tissues (muscle, plasma, kidney, adipose tissue, etc.).

At present, studies of lipid oxidation must take into account both the deterioration of sensorial quality and the appearance of toxic substances. The oxidation process of cholesterol is therefore especially important, since the mutagenic, carcinogenic, and cytotoxic effects of the compounds derived from its oxidation have been clearly demonstrated (*9*−*11*). As well as the initial amount of cholesterol, there are numerous external factors which can influence the formation of cholesterol oxidation products (COPs), among which the most important is the temperature. For this reason, it is difficult to avoid its formation unless the food in question has a very low cholesterol content or none at all (*12*). Some authors express the concentration of COPs in food according to their relationship with the initial cholesterol concentration (*13*), making it necessary to know the total cholesterol content in the samples analyzed.

Both α-tocopherol and cholesterol are components of the unsaponifiable lipid fraction of meat. There are two alternatives for extracting this fraction: the first consists of extracting the total lipid component, saponifying it and later separating the unsaponifiable fraction; the second consists of saponifying the sample directly and later extracting the unsaponifiable fraction. This second option reduces the extent to which the sample is handled and as such reduces the oxidation processes related to extraction (*14*, *15*). The presence of other compounds in important amounts such as squalene and 3-methylpentadecane, and also in lower amounts such as 2-methyltridecane and 2, 4-dimethylundecane in the extract (*16*), is a problem in analyzing α-tocopherol and cholesterol by means of direct measurement. Those artifacts claim for a chromatographic analysis. Gas−liquid chromatography (GLC) has the limitation of not achieving enough separation between α-tocopherol and cholesterol in samples from animal tissues (*17*−*19*), where the amount of α-tocopherol is much lower than cholesterol. Better resolution is achieved when high-performance liquid chromatography (HPLC) is used (*20*). In addition, HPLC analysis of α-tocopherol

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and cholesterol is faster than GLC analysis. By identifying and quantifying α-tocopherol and cholesterol in the extracted fraction using a fluorescence detector and a light-scattering detector, respectively, interference with the solvents and other extracted substances from the sample is avoided (*21*).

The objective of this study is to develop a method for the simultaneous determination of α-tocopherol and cholesterol in fresh pig meat. By determining both compounds in one single process, we will be able to reduce the time taken as well as the cost of the analysis.

MATERIALS AND METHODS

Below a description is offered of the assays designed to optimize the extraction process, achieve correct chromatographic resolution, and evaluate the validity of the modifications proposed by us to the method of Liu et al. (*8*).

Chemicals. The reagents (analytical grade) and solvents (HPLC grade) used were α-tocopherol (Aldrich Chemie GmbH, Steinheim, Germany), cholesterol, α-tocopherol acetate, δ-tocopherol, γ-tocopherol, and 5-α-cholestane (Sigma Chemical Co., St. Louis, MO), butylated hydroxyanisole (BHA) (Acros Organics, Geel, Belgium), ascorbic acid, potassium hydroxide, absolute ethanol (Panreac, Barcelona, Spain), isooctane, and tetrahydrofuran (Scharlau, Barcelona, Spain).

Proposed Modified Method. One gram of meat was weighed and placed in a 15-mL tube with a screw-top. Two hundred fifty milligrams of ascorbic acid and 4 μg of α-tocopherol were added. Later, 7.3 mL of saponification solution, constituted by 11.5% potassium hydroxide in a mixture of absolute ethanol (55% v/v) and water (45% v/v), was added. The air was eliminated from the tube by displacement with nitrogen. It was then shaken so that the ascorbic acid was completely dissolved. Next, the tube was placed in a water bath at 80 °C for 15 min and shaken. After this period of time, it was cooled on an ice bed and 4 mL of previously degasified BHA solution (0.01%) in isooctane was added. This was vigorously mixed for 2 min and left to settle until the separation of phases was observed. An aliquot was filled from the upper phase with Pasteur glass pipets, and 20 μL was injected into the chromatograph.

Instrumentation. The HPLC system was made up of the following: an L-6200 pump (Merck-Hitachi, Darmstadt, Germany); a 360 autosampler and a SFM 25 fluorescence detector both from Kontron Instruments (Milano, Italy); and a Sedex 45 light-scattering detector (S. E. D. E. R. E., Vitry-sur-Seine, France). The other components were a Waters Resolve Guard-Pack Silica precolumn and a Waters Resolve 5 μm Spherical Silica column, 3.9 × 150 mm (Waters, Milford, MA).

Separation and Identification of α-Tocopherol and Cholesterol. The mobile phase comprised a mix of isooctane and tetrahydrofuran, the following was established: 90:10 v/v for 0.5 min, 85:15 v/v for 3 min, and 70:30 v/v for 1 min, before the program finished with a mix of 90:10 for 2.5 min. Throughout the process, there was a constant flow of 1 mL/min. The detection conditions were fluorescence detector at excitation wavelength of 297 nm and emission wavelength of 321 nm; light-scattering detector at 45 °C, with air as the carrying gas at a pressure of 2.1 bar.

Effect of Mobile Phase Composition. To establish the effect of the mobile phase composition on separation, different proportional mixes were tried in isocratic conditions, and triplicate injections of standard constant concentration were performed (5 ppm α-tocopherol and 150 ppm cholesterol). The isooctane:tetrahydrofuran isocratic mixes assayed were 95:5, 90:10, 85:15, 80:20, and 70:30 v/v.

Linearity of the Detector Response and Limit of Detection. The range of linear response of each of the detectors and the limit of detection as well were established from the preparation of a calibration curve for each of the compounds. α-Tocopherol: triplicate standards were prepared between 0.1 and 40 ppm of α-tocopherol in isooctane, from a solution of 50 ppm. Cholesterol: triplicate standards were prepared between 0.25 and 225 ppm of cholesterol in isooctane, from a solution of 250 ppm.

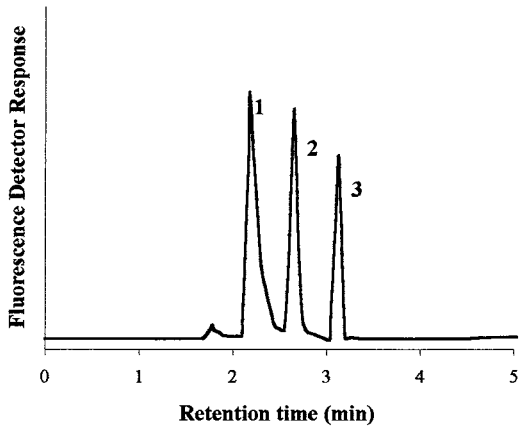

Figure 1. HPLC chromatogram of a mixture of tocopherols. Chromatographic conditions: isocratic mobile phase isooctane:tetrahydrofuran (90:10 v/v); flow rate 1 mL/min. 1: α-tocopherol; 2: γ-tocopherol; 3: δ-tocopherol.

Modification of the Extraction Method. Assays were made of the effect of using a nitrogen atmosphere, the addition of an antioxidant (BHA) and temperature, both during the extraction process and while the extracts were stored. In addition, the effect of each of these factors was determined both individually and in combination on solutions of α-tocopherol in isooctane, after 48 h stored in amber vials at temperatures of 20 °C, 4 °C, and −20 °C. Finally, a study was made of the possible use of α-tocopherol acetate, γ-tocopherol, δ-tocopherol, and 5-α-cholestane as internal standards in the analytical method, by calculating their recovery rates and repeatability.

Determination of Recovery. The recovery capacity of the method was determined on three different concentrations of pure α-tocopherol and cholesterol standards, with and without meat samples. Starting with a solution of 8 ppm α-tocopherol (solution A), and another of 250 ppm cholesterol (solution B), both in ethanol, to each test tube the following volumes were added: 0.25 mL soln A, 0.5 mL soln B; 0.5 mL soln A, 0.75 mL soln B; and 1 mL soln A, 1 mL soln B. The extraction was performed by quadruplicate for each combination under the conditions described in the Proposed Modified Method section.

Repeatability. The repeatability of the extraction procedure, including the proposed modifications, was evaluated by using the average coefficient of variation (CV) of four measurements taken on three mixes of α-tocopherol and cholesterol standards (mix A: 2 ppm α-tocopherol, 125 ppm cholesterol; mix B: 4 ppm α-tocopherol, 175 ppm cholesterol; mix C: 8 ppm α-tocopherol, 250 ppm cholesterol) and on samples of meat added to these three mixes of standards.

Statistical Analysis. For the descriptive and regression statistical analyses, the computer program used was SPSS 10.0 for Windows (SPSS Inc., Chicago, IL). The CV is the ratio standard deviation/average value in percentage.

RESULTS

Selection of the Mobile Phase. The assays performed to optimize the chromatographic method showed that the 90:10 v/v mix (isooctane:tetrahydrofuran) achieved the best resolution of α-tocopherol (retention time of 2.2 min), managing to separate it from other isomers such as δ- and γ-tocopherol (**Figure 1**) and even from the BHA used in the extraction (data not shown). The same phase achieved complete cholesterol elution within 5.8 min. These retention times became shorter when the polarity of the mobile phase was increased (**Table 1**), but using the 70:30 v/v mix other polar compounds extracted from the sample eluted, which can interfere with the correct definition of the cholesterol peak. The elution with isooctane:tetrahydrofuran 70:30 v/v for 1 min was introduced as a washing step. From these results, the gradient described in the Separation and Identification of α-Tocopherol and Cholesterol

Table 1. Effect of Mobile Phase Composition on Retention Time of α-Tocopherol and Cholesterol

mobile phase[a]	α-tocopherol	cholesterol
95:5	3.0[b]	10.4
90:10	2.2	5.8
85:15	1.9	4.3
80:20	1.7	3.3
70:30	1.5	2.5

[a] Isooctane:tetrahydrofuran (v/v). [b] Retention time (min).

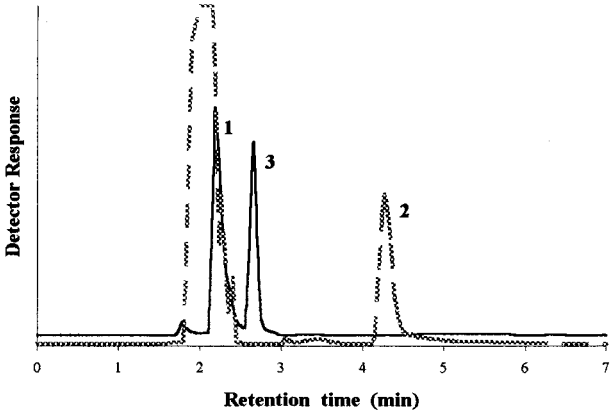

Figure 2. HPLC chromatogram of a muscle tissue sample: (—) fluorescence signal, (---) light-scattering signal. Chromatographic conditions: mobile phase gradient of isooctane:tetrahydrofuran as described in Materials and Methods; flow rate 1 mL/ min. 1: α-tocopherol; 2: cholesterol; 3: BHA.

Table 2. Chromatographic Data for Identifying and Quantifying α-Tocopherol and Cholesterol

	α-tocopherol	cholesterol
calibration curve	$Y = 0.000006\ X$[a]	$Y = 0.0000\ 25X$
CV retention time (%)	0.8	0.9
regression coefficient (r^2)	0.9993	0.9971
retention time (min)	2.2	4.3

[a] Y: concentration (ppm), X: chromatographic area (μvolt × s).

section was established, looking for a good chromatographic resolution of the peaks (α-tocopherol, BHA, and cholesterol) and a retention time as short as possible. The retention times obtained with the gradient method were 2.2 min and 4.3 min (**Figure 2**) for α-tocopherol and cholesterol, respectively, with CV below 1% (**Table 2**). **Figure 2** shows a chromatogram of a sample from pig *Longissimus dorsi* muscle. The whole chromatographic process lasted 7 min, the time necessary for the elution of the two substances under analysis and the stabilizing of the baseline in the light-scattering detector.

Linearity and Limit of Detection. There is high linearity of the chromatographic method for the whole range of α-tocopherol and cholesterol concentrations in meat which are normally found in the literature. The linear response of the detectors was found for concentrations up to 50 ppm for α-tocopherol and 225 ppm for cholesterol (**Table 2**). The regression coefficient (r^2) values were 0.9993 for α-tocopherol and 0.9971 for cholesterol, at a significance level of $P < 0.001$. The limit of detection was found in samples containing 0.1 ppm of α-tocopherol in isooctane and 0.25 ppm of cholesterol in isooctane, which corresponds to 0.4 ppm of α-tocopherol and 1.0 ppm of cholesterol in a sample of meat.

Modifications of the Extraction Method. The use of low temperatures increased the recovery of α-tocopherol. Refrigeration produced a 53% decrease on α-tocopherol loss, being greater (92%) when freezing temperatures were used. The replacement of the air in the headspace of the vial sample by nitrogen reduced the loss of α-tocopherol in 24% at room temperature, being greater at refrigeration temperature (78%) and freezing (58%). The use of BHA yielded similar results. In the presence of BHA, α-tocopherol loss was reduced by 24% at room temperature, being improved in combination of freezing conditions (63%) and refrigeration (71%). The use of a nitrogen atmosphere and BHA in the organic extraction phase had a marked effect on α-tocopherol. The assay on an α-tocopherol solution reduced the losses after 48 h by 31% at room temperature and up to 86% at refrigeration temperatures and 88% when frozen.

Table 3. Rates of Recovery of α-Tocopherol with and without Added Muscle

	with added muscle				without added muscle			
	2 ppm	4 ppm	8 ppm	av	2 ppm	4 ppm	8 ppm	av
recovery[a] (%)	72	65	67	68[b]	91	74	69	78[b]
CV (%)	6.1	5.0	5.8	15[c]	6.0	7.1	4.8	15[c]

[a] Average of quadruplicate analyses for different concentrations of α-tocopherol. [b] Average recovery of the three concentrations analyzed. [c] Average CV of 2, 4, and 8 ppm extracted together.

It was not possible to use any of the substances evaluated (α-tocopherol acetate, γ-tocopherol, δ-tocopherol, and 5-α-cholestane) as an internal standard. α-Tocopherol acetate, which is the derived form of α-tocopherol used for animal dietary supplementation, is not extracted in the organic phase. The γ- and δ- isomers present a high degree of variability in their recovery values, with CV of over 20%. For 5-α-cholestane (retention time of 2.0 min), no chromatographic separation was achieved, which would allow its identification by means of a light-scattering detector. This compound does not emit a fluorescent signal at the excitation and emission wavelengths used in the determination of α-tocopherol.

Recovery and Repeatability. The average recovery of α-tocopherol in standards of different concentrations without added samples of meat was 78% with a CV of 15%. In **Table 3**, it can be seen that at lower concentrations more recovery is achieved (91% for a standard of 2 ppm). The recovery capacity decreased when a meat sample was added to the standards, going down to 68% with a CV among all the concentrations assayed of 15%. The use of a nitrogen atmosphere during extraction, BHA in the organic phase, and frozen storage of the extracts increased by 8.3% the α-tocopherol recovery. The average recovery of the different cholesterol standards was 97% (CV = 6.1%), the highest recovery being found at the lowest concentration of those assayed (**Table 4**). In the assays performed with meat samples, an average recovery of 105% (CV = 3.6%) was achieved.

The repeatability of the chromatographic method in determining α-tocopherol and cholesterol was high. The CV achieved was 5.6% (average of 6.1, 5.0, and 5.8, **Table 3**) for α-tocopherol in mixtures of standards and meat and 5.9% (average of 7.6, 3.3, and 6.7, **Table 4**) for cholesterol. This method was used for the simultaneous analysis of α-tocopherol and cholesterol in samples of *Longissimus dorsi* muscle from twelve different pigs. The average amount of α-tocopherol determined was 1.8 mg/kg of fresh meat (CV of 14%). The amount of cholesterol determined was 620 mg/kg of fresh meat (CV of 5%).

Table 4. Rates of Recovery of Cholesterol with and without Muscle

	with added muscle				without added muscle			
	100 ppm	150 ppm	200 ppm	av	100 ppm	150 ppm	200 ppm	av
recovery[a] (%)	104	110	102	105[b]	104	95	92	97[b]
CV (%)	7.6	3.3	6.7	3.6[c]	4.1	3.1	6.7	6.1[c]

[a] Average of quadruplicate analyses for different concentrations of cholesterol. [b] Average recovery of the three concentrations analyzed. [c] Average CV of 100, 150, and 200 ppm extracted together.

DISCUSSION

The method described in this study makes it possible to quantify the α-tocopherol and cholesterol content in fresh pig meat in just one analytical process. The extraction technique used is a modification of that of Liu et al. (*8*), who in turn simplified the method of Arnold et al. (*7*). This consists of a direct saponification technique without previous fat extraction, thus reducing the duration of the analysis and the oxidation of the sample (*15*).

By means of the modifications made to the process (the use of BHA in the organic solvent and a nitrogen atmosphere during processing), an increase of 5% was achieved in the extraction level of the α-tocopherol standard added to the sample, reaching 68% recovery. The extraction capacity in pure standards (91%) was close to the results presented by Liu et al. (*8*), who obtained a level of 94% in the assay prepared with 3 μg of standard. The differences between extraction capacity in pure standards and in standards added to a sample were around 10%, which is comparable to that described in the original method by Arnold et al. (*7*). The degree of variation in the α-tocopherol recovery capacity (CV = 15%) and the fact that it was impossible to use another compound as an internal standard obliged the authors to add a known quantity of α-tocopherol to a duplicate of the sample to determine how much was recovered. The direct saponification method used was shown to be effective for extracting cholesterol (close to 100%), as was found by Al-Hasani et al. (*14*), who obtained a high level of correlation (r = 0.9996) between a direct saponification method and the official cholesterol determination method (*22*), which was based on previous extraction of the complete lipid fraction. The α-tocopherol and cholesterol CV obtained (**Tables 3** and **4**) were comparable to those found by Liu et al. (*8*) and Pfalzgraf et al. (*23*).

The chromatographic conditions described in the method made it possible to resolve α-tocopherol and cholesterol correctly and quickly from other substances extracted at the same time from the sample, thus reducing the retention time for α-tocopherol as published by Arnold et al. (*7*), who uses a 96:4 v/v mixture of isooctane:tetrahydrofuran, and by Pfalzgraf et al. (*23*) with a mixture of hexane and ethyl acetate (95:5 v/v).

The serial combination of the fluorescence detector and the light-scattering detector used by us made it possible to identify α-tocopherol and cholesterol in just one analytical process, as previously Murphy et al. (*21*) had done. These authors used various detectors placed in series to detect certain compounds in cell cultivation. Those detectors were sensitive enough to analyze the concentrations of these substances which are described in the literature (*24, 25*). Although we did not analyze real samples, neither in other chromatographic conditions (e.g., higher retention times for α-tocopherol) nor with a mass spectrometer detector, to verify a possible overlapping of signals, it should be considered that under the wavelengths of excitation and emission used in the fluorescence detector, only the α-tocopherol from the sample and the BHA can be detected. To interfere, other compounds present in the sample having the same fluorescence characteristics should coelute with α-tocopherol, which is not the case (*21*). In the chromatograms shown in **Figure 2**, there is no overlapping of the signal of α-tocopherol, since those are two different chromatograms, those peaks being around 2 min of retention time, effectively at the same time, but not in the same place. Each one is at the respective detector.

The correlation range in the calibration curve standards (0.1−50 ppm of α-tocopherol and 0.25−225 ppm of cholesterol) allows to analyze in a range of concentrations from 0.4 to 200 ppm of α-tocopherol (0.4−200 mg α-tocopherol/kg of meat) and from 1 to 900 ppm cholesterol (1−900 mg cholesterol/kg of meat). These ranges are broad enough to be used in fresh and cooked pig, cow, goat, and poultry meat. For cholesterol, no internal estandard was used since it has a high recovery (around 100%) and a low CV. Both compounds are finally quantified using the respective calibration equation and the amount of internal standard employed, if any.

The amount of α-tocopherol determined in samples from *Longissimus dorsi* is similar to the values reported by Asghar et al. (*3*) and Monahan et al. (*2*), 0.5 and 3.2 ppm, respectively, in pigs fed with diets supplemented with similar amounts of α-tocopherol acetate. The CV reported is similar to that found by us using this modified method. The amount of cholesterol determined is within the broad range of values described in the literature (*24*).

In conclusion, the analytical method described makes it possible to determine accurately α-tocopherol and cholesterol concentrations in meat by using just one analytical process lasting 7 min. The direct saponification extraction method has been shown to be efficient in achieving high repeatable recovery percentages for both substances. The modifications introduced to the original method brought about an increase in recovery percentages. The detectors used proved to be sensitive enough to identify the concentrations described in the literature for these substances.

ABBREVIATIONS USED

BHA: butylated hydroxyanisole; COPs: cholesterol oxidation products; CV: coefficient of variation; LSD: light-scattering detector.

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