Estimation of Lipoyllysine Content in Meat and Its Antioxidative Capacity

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ABSTRACT: During this research a simple, accurate, and environmentally friendly method to determine lipoyllysine and lipoic acid in meat was developed and validated. The presented approach was based on the hydrolysis of the proteins containing lipoic acid, reduction of disulfide bonds with tris(hydroxymethyl)phosphine, and precolumn derivatization of free thiol groups with 1-benzyl-2-chloropyridinium bromide long-term followed by HPLC separation with a diode-array detector. The method has been validated in accordance with the U.S. FDA guidelines and was linear in the range of 0.1−10 μmol/L in concentration with R² values ≥0.9997 for both analytes. For lipoyllysine and lipoic acid, intra- and interday precision values were lower than 10%. The intraday accuracy values ranged from 91.0% to 99.4% for lipoyllysine and from 99.1% to 107.3% for lipoic acid, whereas the interday accuracy values for lipoyllysine and lipoic acid were 92.0−95.6% and 93.5−98.8%, respectively. Additionally, in this research the antioxidant activity of lipoyllysine and reduced lipoic acid compound was examined by spectrophotometric method with 1,1-diphenyl-2-picrylhydrazyl. The data showed that dihydrolipoyllysine exhibits stronger antioxidant capacity than lipoyllysine based on a lower value of concentration required to achieve a 50% antioxidant effect in the 1,1-diphenyl-2-picrylhydrazyl radical scavenging test.

KEYWORDS: antioxidants, 2,2-diphenyl-1-picrylhydrazyl, high performance liquid chromatography, hydrolysis, lipoyllysine, radical scavenging, tissue

INTRODUCTION

For the past few years, medical research has shown that healthy diets and long-term consumption of foods rich in antioxidants may lower oxidative stress and the occurrence of chronic diseases, such as cancer and diabetes, as well as neurodegenerative and cardiovascular diseases.1,2

In regular and healthy people, there is an equilibrium between endogenous antioxidant defenses and the generation of reactive oxygen species (ROS) or free radicals. However, if this balance is disturbed, it can lead to oxidative stress and related damage. Consequently, oxidative stress damages proteins, lipids, and nucleic acids, thus compromising cell viability.3,4 To minimize the harmful effects of free radicals, organisms are endowed with a very effective antioxidant defense system. Natural antioxidant enzymes (catalase, superoxide dismutase, horseradish peroxidase) and nonenzymatic antioxidant compounds, such as vitamins E and C, α-lipoic acid (LA), and glutathione, inhibit the oxidative mechanisms that lead to the development of degenerative diseases and aging.5,6 Because oxidative damage of human cells increases with age, the intensified ingesting of exogenous antioxidants derived from fruits, vegetables, and meat may assist the endogenous antioxidant defense system and thus reduce the risk of chronic diseases.6

LA belongs to a group of compounds absorbed from food and it is widely known as the “universal antioxidant” and “antioxidant of antioxidants.”7−9 LA and dihydrolipoic acid (DHLA), which is a reduced form of LA, meet all of the criteria for a perfect antioxidant. They can easily scavenge ROS, chelate metals, and do not present any serious side effects. They can also interact with other antioxidants and can regenerate them.10,11 It should be emphasized herein that despite the large number of naturally occurring antioxidants the search for new, natural compounds with antioxidant activities remains a growing research area. Numerous methods for the antioxidant activity assessment have been described in the review by Pisoschi and Negulescu.12 In food and biological samples, LA occurs mainly in a protein-bound form through the amide linkage to the ε-NH₂ group of lysine and the carboxylic group of LA.6,13 The protein-bound form, lipoyllysine (LLys), plays an important role in oxidative metabolism as a part of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complex, which controls the Krebs cycle, ultimately leading to the production of adenosine triphosphate.14,15 Since the measurement of LLys is not an easy analytical task, only limited information exists about LLys concentration in tissues. Liberation of LA linked to proteins required the use of a hydrolysis step of the samples (often under a strong acid or alkaline conditions) and solvent extraction procedure. Therefore, this results in low recoveries because LA can experience considerable decomposition.

There are several methods for LA or LLys quantification in biological tissues (Table 1), which include the colorimetric
The most commonly consumed samples of liver, heart, kidney, and stomach were further treated with 15% 3 mol/L PCA and 200 μL of MeCN were added. The mixture was carefully stirred and heated at 40°C for 20 min. After the reaction, 20 μL of hydrochloric acid (3 mol/L) was added to the solution to decompose an excess NaBH₄. At last, the mixture was cooled down to room temperature. After the reaction, LA was prepared in 0.1 mol/L NaOH, 0.01 mol/L LLys was prepared in 1 mol/L HCl, while 0.1 mol/L BCPB was prepared in deionized water. No noticeable change in analyte content was reported for these solutions at 4°C by HPLC method. The working solutions were prepared daily through dilution with deionized water. Fresh solutions of THP (0.25 mol/L) and NaBH₄ (1 mol/L) were prepared daily in deionized water. A stock solution of 0.001 mol/L DHLA was obtained by mixing 100 μL of 0.01 mol/L LA and 200 μL of 1 mol/L NaBH₄. The mixture was carefully stirred and heated at 40°C for 20 min. Next, the mixture was cooled down to room temperature. After the reaction, 20 μL of hydrochloric acid (3 mol/L) was added to the solution to decompose an excess NaBH₄. At last, the mixture was diluted with deionized water to a final volume of 1 mL. Similarly, a solution of 0.001 mol/L dihydrolipoyllysine (DHLlys) was obtained.

A fresh stock solution of Pronase E (activity of 36 U) was obtained by mixing 1.8 mg of enzyme with 2 mol/L LaCl₃ prepared in borane buffer (0.1 mol/L, pH 8). Working standard solutions of Pronase E were obtained daily by appropriate dilution of the stock solutions in 0.1 mol/L CaCl₂, every day and were processed immediately. Commercially available subtilisin A is present in the form of aqueous solution and was kept at 4°C until processing. Sample Preparation. Homogenization. One gram of animal tissue was mechanically blended in 10 mL of 0.1 mol/L cold borate buffer at pH 8 in Falcon tubes for 30 s to 1 min (at an operating speed of 24 000 rpm) using a high-powered rotor-stator homogenizer.

Enzymatic Hydrolysis of LA and LLys. Two hundred microliters of each homogenate was suspended in the amount of proteases (subtilisin A and Pronase E) recommended for the selected animal tissues (see SI Table S1). The samples were then vortexed and incubated at 37°C for 22 h in stoppered Eppendorf tubes. Determination of LLys. After enzymatic digestion, the tissue samples were further treated with 15 μL of 0.25 mol/L THP for 5 min to reduce S−S bonds. In the next step, 10 μL of 0.1 mol/L BCPB was added to begin the derivatization reaction, during which the samples were vigorously mixed and put aside at room temperature for 15 min. To separate LA and LLys derivatives from the protein, 20 μL of 3 mol/L PCA and 200 μL of MeCN were added. The mixture was subsequently vortex-mixed and set aside for 5 min and finally centrifuged for 10 min (14 000g, 4°C). A 5 μL solution from the above protein was transferred to the HPLC system.

Chromatographic Analysis. The LA and LLys derivatives were separated from other compounds by the use of mobile phase containing 2% acetic acid solution, pH 2.5, and MeCN in the gradient mode as follows: 0–6 min, 9–21% (MeCN); 6–11 min, 21–30% assay,16 an enzymatic method,17,18 gas chromatography,19–23 and high performance liquid chromatography (HPLC).15,24–25 However, only three HPLC methods, based on ultraviolet,24 electrochemical,25 and fluorescence detections,15 have been developed in recent years. Although these methodologies appear to be appropriate for the detection of LLys, the utility in biological samples is not sufficient enough in terms of selectivity and sensitivity. Furthermore, it has not been determined whether LLys possesses antioxidant properties or whether it may act as a source of free LA.15,26 Moreover, there is still a lack of detailed information about LLys level in animal tissues.15,17 Consequently, the aim of this study was to develop a new method that would allow for the simultaneous quantification of LLys and LA in meat samples as well as to examine LLys antioxidant activity by using a spectrophotometric method with 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Table 1. Overview of Analytical Methods, Parameters, and the Content of Lipoic Acid (LA), and Lipolysine (LLys) in Meat Products

| method               | sample          | analyte | linear range      | recovery | LOD  | LOQ  | reference |
|----------------------|-----------------|---------|-------------------|----------|------|------|-----------|
| colorimetric assay   | animal tissues  | LA      | 0−100 μmol/L      | NR       | NR   | NR   | 16        |
| enzymatic assay      | animal tissues  | LLys    | 1−5 μmol/L        | NR       | 0.1μmol/L | 17    |
| GC-FID               | chick livers, chicken eggs | LA | NR | 34% | NR | 21 |
| GC-FID               | bacteria, cow’s milk, rat liver, kidney | LA | 0−97 μmol/L | >87% | NR | NR | 22 |
| GC-MS                | animal tissues  | LA      | 0.05−97 μmol/L    | 60−70%   | 0.05μmol/L | NR | 20 |
| GC-FDP               | mouse tissue, bacterial cells | LA | 20−500 ng | 50−94% | 50 pg | NR | 19 |
| HPLC-FLD             | spinach, animal samples | LLys | 0.015−1 μmol/L | 99−107% | 0.007 μmol/L | 0.022 μmol/L | 15 |
| HPLC-ECD             | plant and animal tissue | LLys | 0−2.5 pg/g | 100% | NR | NR | 25 |
| HPLC-UU              | enzyme hydrolysates | LLys | 117−120% | NR | NR | 24 |
| HPLC-DAD             | liver, heart, kidney and stomach | LA LLys | 0.1−10 μmol/L | 88−101% | 0.03 μmol/L | 0.1 μmol/L | proposed method |

*NR: Not reported.
The flow rate was 1 mL/min and the column temperature set to 25 °C. The detection wavelength was 321 nm. Identification of LLys-BCPB and LA-BCPB peaks was based on a comparison of retention times and DAD spectra with a similar set of data obtained from standard solutions.

**Analytical Method Validation.** The proposed method was validated under optimized experimental conditions in accordance with U.S. FDA guidelines. The applied protocol involved an evaluation of the selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision, matrix effect, and stability of LA and LLys derivatives.

**Selectivity.** The method selectivity was evaluated after analysis of six pork liver and turkey heart homogenate samples obtained from different sources. Investigation of the potential interferences at the region assigned to chromatographic peaks of the LA and LLys derivatives determined selectivity. The procedures for sample preparation and chromatographic conditions described above were used. The peak purity test using a DAD was also executed.

**Detection and Quantification Limits.** The LOD and LOQ were calculated considering the parameters of the analytical curves performed for turkey heart homogenates with the use of equations:

$$\text{LOD} = 3 \frac{S_a}{b} \quad \text{and} \quad \text{LOQ} = 10 \frac{S_a}{b},$$

where $S_a$ is the standard deviation of the y-intercept of the calibration curve and $b$ is the slope of the regression line.

**Linearity.** To prepare the calibration standards for the determination of LLys and LA in meat homogenates, portions of 200 mL of turkey heart homogenate were each transferred to tubes. To each tube, increasing amounts of working standard solution of LLys and LA was added. The final concentrations of analytes were between 0.1 and 10 μmol/L homogenate. The calibration standards were then hydrolyzed and processed in triplicate, according to procedures presented in Sample Preparation.

The peak heights of LLys and LA as 2-S-pyridinium derivatives were plotted versus the analyte concentration and the equations of the calibration curves were obtained using least-squares linear regression.

**Precision and Accuracy.** Accuracy and precision of the analytical method were determined by the addition of known amounts of LLys and LA to turkey heart. Three concentrations in the entire range of the calibration curves were studied: one near the LOQ, one near the center and one near the upper end of the calibration curves. The enriched meat samples were handled according to the analytical procedure presented in Sample Preparation. For an intraday study, the analyses were performed on five turkey heart samples on the same
day, and for an interday study the analyses were performed in triplicate on five consecutive days.

Matrix Effect. The matrix effect was studied with calibration standards prepared in standard aqueous solutions and in meat homogenate. The curves of aqueous solutions were prepared as described for the linearity assessment. Then the slopes of the two calibration curves were compared for each of the analytes studied. The matrix effect was evaluated using the following equation

\[
\text{The matrix effect} = \left( \frac{A}{B} - 1 \right) \times 100
\]  

where \( A \) and \( B \) are the slope of calibration curves in matrix and in aqueous solution, respectively.

Stability of the 2-S-Pyridinium Derivatives. The short-term stability of 2-S-pyridinium derivatives of LLys and LA in turkey heart homogenate were tested. Ten nanomoles/milliliter of LLys and LA in turkey heart homogenate were prepared and processed according to the procedure described in Sample Preparation. Acidified solutions from the above protein containing MeCN (\( n = 2 \)) were placed in an autosampler at room temperature and analyzed at time zero and every hour for 28 h.

DPPH Radical Scavenging Activity. The antioxidant activities for different standard compounds (LA, LLys, DHLA, and DHLlys) were measured using the DPPH free radical scavenging assay according to the modified methods reported by Mishra et al. and Madawala et al.\(^7,31\) Briefly, 3 mL of the antioxidant solution was mixed with 3 mL of 60 \( \mu \)mol/L methanolic solution of DPPH. The reaction tubes, in pairs, were shaken vigorously and left in the dark at room temperature for 30 min. The control sample was a methanolic solution of DPPH with deionized water. The decrease in absorbance at 520 nm. All measurements were done under dim light. DPPH radical scavenging capacity was calculated using the following equation

\[
\text{DPPH radical scavenging activity (\%) = } \left( \frac{A_0 - A_i}{A_0} \right) \times 100
\]  

\( A_0 \) and \( A_i \) are the absorbance at 520 nm of the DPPH radical in the presence and absence of antioxidant, respectively.

Different sample concentrations (expressed as the number of moles of antioxidant/mol DPPH) were used to determine the antiradical curves for calculating the EC\(_{50}\) values defined as the amount of antioxidant required to decrease the initial DPPH concentration by 50%. The curves were plotted referring to concentrations on the x-axis and their percentage of DPPH radical remaining against the sample concentration on the y-axis. The EC\(_{50}\) value (\( \mu \)mol/L) was calculated using the linear interpolation method.

# RESULTS AND DISCUSSION

Sample Preparation. Sample preparation has an impact on nearly all of the subsequent analysis steps and is hence crucial for the unequivocal identification, confirmation, and quantification of analytes. This is more critical for analytes present at trace content in complex matrices.

It was found that LA in food and biological samples occurs mainly in a protein-bound form. As a result, the measurement of LLys is not an easy analytical task and proper optimization of the sample preparation procedure is essential before their assay. In earlier studies, applying strong acid or alkaline conditions to liberate LA from the samples, followed by solvent extraction from the hydrolysate resulted in low recoveries because LA was considerably decomposed. On the other hand, GC methods mentioned in the review by Kataoka\(^26\) offered the possibility to determine the sum of the free LA and LLys, but it was difficult to distinguish the free LA from the protein-bound LA. To counteract the above difficulties, the application of an enzymatic hydrolysis is recommended. Several enzymes, such as Prone E and subtilisin A, were used to determine the protein-bound LA and free LA. Different incubation times and amounts of enzymes were tested to obtain the most favorable conditions. According to the literature data,\(^32\) enzymatic cleavage should be carried out in the presence of calcium chloride in the pH range 5.0 to 9.0. Under these conditions, enzymes are highly stable, and the rate of hydrolysis is considerably higher. Our studies show that when the reaction was carried out at a physiological temperature of 37°C and under mild alkaline conditions (pH 8.0), the reaction reached a plateau after 22 h (data not shown) using the appropriate amounts of proteases (subtilisin A and Prone E), which were recommended for the selected animal tissue (SI Table S1).

In the next step, the disulfide bonds (\(-S-S-\)) in free LA and LLys liberated during the enzymatic digestion were reduced with THP to their thiol forms (\(-SH\)) and derivatized with BCPB (Figure 1). Investigations on the reduction conditions showed the best yield was obtained when the reaction proceeded at room temperature for 5 min. We observed that the use of THP as a reducing agent showed many advantages over another studied tris(2-carboxyethyl)phosphine reagent.\(^14\) In particular, by using THP the reduction reaction was carried out faster and was characterized by a high efficiency. The derivatization reaction occurred under mild conditions (room temperature, pH 8, 15 min). Additionally, the derivatization conditions of the proposed method were favorable compared to another previously published study utilizing fluorescence detection.\(^35\) The reduced LLys was fluorescently labeled with ammonium 4-fluoro-2,1,3-benzoxadiazole-7-sulfonate at 60°C for 1 h, whereas the results of the proposed method indicated total derivatization occurred within 15 min at room temperature.

As with plasma samples,\(^33\) a crucial step in the preparation of animal tissues is the separation of analytes from the protein. Meat tissue is difficult to analyze because of its high content of unsaturated fatty acids. Moreover, LA and LLys are amphiphilic molecules which can interact with the surface of proteins. In the final step of sample preparation, a deproteinization procedure has been used. However, as evidenced by recent studies,\(^33\) acidic deproteinization of plasma proteins markedly adsorbs LA. As such, the analyte can be accidentally removed with the precipitated proteins and the concentration of LA in the solution becomes lower than expected. In this study, we also observed a lower LLys and LA concentration in the final analytical solutions for meat homogenates deproteinized with PCA compared to the standard solution. The recoveries of LLys and free LA were calculated using the following formula

\[
\text{Recovery (\%) } = \frac{\text{analyte amount in homogenate sample}}{\text{analyte amount in standard solution}} \times 100
\]  

The recovery values for LLys and free LA were 80.7% and 44.3%, respectively, meaning that approximately 20% of LLys and 56% of free LA were adsorbed on proteins. Better results were obtained when a mixture of PCA and MeCN was used; the recoveries reached 100%. Hence, for deproteinization of meat homogenates, the additional mixture of PCA and MeCN is recommended during the determination of LLys and LA.

Chromatographic Analysis. Different chromatographic conditions were carefully studied and optimized to achieve good separation of the 2-S-pyridinium derivatives of LLys and
LA from each other and from the endogenous matrix components within a suitable run time. For this purpose, several parameters, such as the amount of organic modifier, the acetic acid concentration, and various gradient elution, were tested. The optimum HPLC conditions were described in Chromatographic Analysis. Under the chosen chromatographic conditions, LLys showed a retention time of 6.45 (±0.04; n = 6) min and LA showed a retention time of 8.79 (±0.06; n = 6) min. The representative chromatogram of LLys and LA in the turkey heart homogenate is shown in Figure 2. The total time of the analytical run was 16 min. Before analyzing the samples, six consecutively replicated analyses of the meat were assessed in order to investigate the suitability parameters including the retention factor, the asymmetry factor, and the number of theoretical plates. The results showed a satisfactory system efficiency, which generated narrow peaks. As can be seen in the chromatogram, the ability to accurately measure the analyte of interest in the presence of the additional components in the sample matrix. To demonstrate the selectivity of our method, six different samples of pork liver and turkey heart homogenates with and without spikes in LLys and LA were compared. HPLC analysis of the derivatized hydrolysates showed no interfering peaks with the signals obtained for the analytes. LLys-BCPB and LA-BCPB peaks were well separated from other peaks of endogenous and extraneous substances, as depicted in the representative RP-HPLC chromatograms in Figure 2. Moreover, examination of peak purity showed that peaks assigned to LLys and LA derivatives were not attributable to more than one component.

Analytical Method Validation. Selectivity. Selectivity is the ability to accurately measure the analyte of interest in the presence of the additional components in the sample matrix. To demonstrate the selectivity of our method, six different samples of pork liver and turkey heart homogenates with and without spikes in LLys and LA were compared. HPLC analysis of the derivatized hydrolysates showed no interfering peaks with the signals obtained for the analytes. LLys-BCPB and LA-BCPB peaks were well separated from other peaks of endogenous and extraneous substances, as depicted in the representative RP-HPLC chromatograms in Figure 2. Moreover, examination of peak purity showed that peaks assigned to LLys and LA derivatives were not attributable to more than one component.

Detection and Quantification Limits. The LODs for LLys and LA were 0.1 μg/g (0.03 μmol/L) and 0.06 μg/g (0.03 μmol/L), respectively, whereas LOQs for LLys and LA were 0.33 μg/g (0.1 μmol/L) and 0.21 μg/g (0.1 μmol/L), respectively. The LOD value achieved with this assay for LLys was worse than obtained by the HPLC method with fluorescence detection and three times lower than the previously reported method. The LOD for LA was comparable with the previously published GC method with MS detection as was presented in Table 1.

Linearity. For studied meat samples, seven-point calibration plots were built for LLys and LA in triplicate. A linear relation between peak height and concentration of analyte was observed in the tested ranges for LLys and LA in all meat homogenates. As the slopes of the calibration curves were not significantly different, the validation method was performed for only one tissue, turkey heart. For turkey heart homogenate samples, the linear regression equations were \( y = 0.348x + 0.2945 \) for LLys and \( y = 0.4842x + 0.0915 \) for LA. The coefficient of correlation (R²) of the analytical curves was 0.9997 and 0.9999 for LLys and LA, respectively.

Precision and Accuracy. Three concentrations, that is, 0.1, 1, 10 μmol/L, were used to prove the precision and accuracy of the developed method. The precision was expressed as the relative standard deviation (RSD), whereas the accuracy was expressed as the percentage of analyte recovered. Accuracy was calculated using the equation

\[
\text{Accuracy} \, (\%) = \frac{\text{measured amount} - \text{endogenous content}}{\text{added amount}} \times 100
\]

Table 2. Estimation of the Intraday and Interday Precision and Accuracy for Lipoyllysine (LLys) and Lipoic Acid (LA) in Meat Samples (n = 5)

| analyte | added (μmol/L) | founded ± SD (μmol/L) | precision (%) | accuracy (%) |
|---------|----------------|------------------------|--------------|-------------|
|         | intraday       | interday               | intraday     | interday    |
| LLys    | 0.1            | 1.06 ± 0.05            | 4.7          | 91.0        |
|         | 1              | 1.86 ± 0.07            | 3.9          | 97.7        |
|         | 10             | 10.82 ± 0.44           | 4.1          | 99.4        |
|         | 0.1            | 0.36 ± 0.01            | 3.3          | 99.1        |
| LA      | 1              | 1.23 ± 0.05            | 4.2          | 107.3       |
|         | 10             | 10.19 ± 0.43           | 4.2          | 100.3       |

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Results of intra- and interday accuracy and precision are summarized in Table 2. The values obtained in our study meet the acceptance criteria since RSD did not exceed 20% for LOQ and 15% at all concentration levels. Moreover, the results obtained indicate that our method provides very good accuracy and precision. Therefore, this method can improve upon the shortcomings associated with poor accuracy and precision values for LLys in animal tissues, which were formerly obtained by the HPLC methods with fluorescence and ultraviolet detections.15,24

The Matrix Effect. Sample matrix effect was studied by comparing the slopes of the calibration curves of the standard aqueous solutions with the slopes obtained when analyzing the spiked samples. The matrix effect for LLys and LA was lower than ±20%, which denotes the absence of any matrix effect and confirms that the meat homogenate matrix does not interfere with signals of the analytes.

Stability of the 2-S-Pyridinium Derivatives. As we have previously demonstrated,33 LA-BCPB in plasma was stable at room temperature for 4 h. However, in meat homogenate samples the 2-S-pyridinium derivatives of both LA and LLys were found to be stable at ambient temperature for a much longer time. No significant change in peak height was noted when the acidified samples containing MeCN were kept in the autosampler for 24 h (data not shown). The residual percentages of LLys and LA after this time were 104.5% and 106.4%, respectively.

DPPH Radical Scavenging Activity. DPPH is commonly used to estimate the free radical scavenging ability of pure compounds. It is a stable free radical, which possesses an unpaired valence electron on one atom of the nitrogen bridge.14 By accepting a hydrogen atom from an antioxidant, DPPH is transformed into DPPH-H and the color of the solution changes from purple to yellow with an associated decrease in absorbance at 515–520 nm.31 This color change is measured spectrophotometrically and used to estimate the parameters for antioxidant properties.

In the present study, LA, LLys, and their reduced forms, that is, DHLA and DHLLys, were analyzed to evaluate the free radical scavenging capacity of these compounds. Each compound was tested in the range of 2.5–60 μmol/L. The concentrations were chosen based on their antiradical capacity toward the DPPH radical based on pilot determinations and literature data.

Results suggest that LA and LLys did not scavenge the DPPH radical, even at higher concentrations (200–2000 μmol/L), despite LA being used in numerous in vivo studies with promising results and being known as the “universal antioxidant”. In the case of LLys, it has not been clearly identified whether LLys possesses any antioxidant activity or whether it acts as a source of free LA.15,26 Nevertheless, in our studies we observed that DHLLys can scavenge DPPH radical (Figure 3), although at a lower scavenging capacity, as compared to DHLA. DHLA and DHLLys have stronger antioxidant activity due to their two free thiol (−SH) groups than the dithiolenes.35 EC50 values for DHLA and DHLLys were calculated as 0.076 μmoles DHLA/μmoles DPPH (EC50 4.56 μmol/L) and 0.094 μmoles DHLLys/μmoles DPPH (5.64 μmol/L), respectively. Thus, the measured EC50 for DHLA is lower than the earlier reported value of 0.39.7

Application of the Method. The developed method was applied to monitor LLys and LA concentrations in five different cow, calf, pig, chicken, and turkey samples (including liver, heart, kidney, and stomach). Heart, liver, and kidney showed the highest levels of LLys and LA among all of the examined tissues. The mean amounts of LLys were in the range of 2.11–3.99 μg/g for heart, 0.56–1.17 μg/g for liver, and 0.71–1.40 μg/g for kidney, whereas the amounts of LA ranged from 0.22 to 0.55 μg/g for heart, from 0.38 to 0.51 μg/g for liver, and from 0.14 to 0.50 μg/g for kidney. LLys was also found in cow and pig stomachs, while LA was only found in chicken stomachs. Results of LA and LLys concentrations in all assayed samples are shown in Table 3. Considering our results described above and the available literature data, it may be concluded that LLys content is high in metabolically active tissues, which contain a high population of mitochondria.36

The tissue level of free LA was relatively low, if present at all. This indicates that proteolytic enzymes do not effectively cleave an amide bond between LA and a protein lysine. Therefore, after digestion, LA is absorbed as LLys.17 The concentrations of LA and LLys also seem to be animal species dependent. In chicken meat, the lowest amounts of LA and LLys were detected, whereas in red meat the concentrations of LA and LLys were significantly higher. It seems that the high levels of LA and LLys in tissues such as heart, liver, and kidney may provide an antioxidant defense system for these organs.

We observed some differences in the LLys concentrations found in tissue samples examined using the present method and other previously reported methods.17,25 This discrepancy may be due to the physical state of the sample (e.g., fresh sample or acetone powder), the animal species (e.g., bovine, calves, pigs, chicken, and turkey), and differences in nutrition, age, sex, and, primarily, the methods used (e.g., enzymatic, GC, ECD). The LLys contents in bovine tissue acetone powders by the HPLC-ECD method25 were determined for kidney, heart, and liver tissues and were from 0.86 to 2.64 μg/g. The values obtained in the present study are somewhat higher compared to these values as well as lower in comparison to the values previously published utilizing the enzymatic method (from 2.14 to 4.38 μg/g for the same tissues).17

The determination of the LLys in the homogenates of calf, pig, chicken, and turkey samples (liver, heart, kidney, and stomach) has not yet been reported and seems to be the first example.

In summary, a new, simple and selective method for the simultaneous quantification of LLys and LA in meat using the HPLC technique with DAD detection has been described and validated. To the best of our knowledge, the present article is currently the first report in which the examination of LLys antioxidant activity through a spectrophotometric method with DPPH was performed. Our new approach includes a handy analytical protocol for the determination of LLys and LA,
which is characterized by the following advantages over previous methodologies: (i) it does not require the use of drastic hydrolysis conditions to release LA from the samples, which may result in partial LA degradation and low recovery; (ii) the derivatization reaction takes place at room temperature as opposed to the higher temperature required in thermally initiated derivatization; (iii) the short chromatographic run time reduces the consumption of solvents and generates small waste amounts; (iv) the developed analytical scheme is able to determine LLys and LA in meat samples with adequate waste amounts; (v) the validation parameters are within the criteria of analysis for biological samples; (vi) this method is easily accessible to a majority of analytical laboratories and does not require particularly expensive maintenance and thus represents an excellent analytical tool alternative for more complicated chromatographic instruments (HPLC-FLD, HPLC-ECD).

The determination of LLys and LA in biological and food samples is important in the study of biochemical reactions, nutritional and pharmacodynamic studies, and the diagnosis of certain types of diseases. Furthermore, there is still a lack of knowledge of the LLys role in the organism and its pharmacological benefits. Thus, in our opinion it was interesting to get more detailed quantitative data about its content in meat.

We believe that our innovative studies on the antioxidant properties of LLys may pave the way for the new generation of pharmaceutical preparations. This study is also expected to benefit researchers working with new antioxidants.

Table 3. Content of Lipoic Acid (LA) and Lipoyllysine (LLys) in Edible Animal Products

| tissues   | turkey µg/g tissue | call µg/g tissue | cow µg/g tissue | pig µg/g tissue | chicken µg/g tissue |
|-----------|--------------------|----------------|-----------------|----------------|-------------------|
| LLys      |                    |                |                 |                |                   |
| heart     | 3.99 ± 0.01        | 3.32 ± 0.01    | 3.12 ± 0.05     | 3.46 ± 0.03    | 2.11 ± 0.00       |
| liver     | n.d.               | 0.56 ± 0.00    | 0.97 ± 0.01     | n.d.           | 1.17 ± 0.01       |
| kidney    | n.d.               | 0.71 ± 0.01    | 1.40 ± 0.01     | 0.94 ± 0.01    | 0.72 ± 0.01       |
| stomach   | n.d.               | n.d.           | 1.59 ± 0.02     | 0.54 ± 0.01    | n.d.              |
| LA        |                    |                |                 |                |                   |
| heart     | 0.36 ± 0.01        | 0.22 ± 0.01    | 0.28 ± 0.04     | 0.55 ± 0.01    | 0.27 ± 0.01       |
| liver     | n.d.               | 0.38 ± 0.00    | n.d.            | n.d.           | 0.51 ± 0.00       |
| kidney    | n.d.               | 0.14 ± 0.03    | 0.50 ± 0.05     | 0.41 ± 0.00    | 0.32 ± 0.01       |
| stomach   | n.d.               | n.d.           | n.d.            | n.d.           | 0.41 ± 0.03       |


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Author Contributions
A.K. designed the experiment, collected samples, performed the experiment, analyzed the data, and wrote original draft of the manuscript; G.C. collected samples, gave critical comments and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviation Used
BCPB, 1-benzyl-2-chloropyridinium bromide; DHLA, dihydrolipoic acid; DHLlys, dihydrolipoyllysine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; LA, α-lipoic acid; LLys, lipoyllysine; MeCN, acetonitrile; PCA, perchloric acid; ROS, reactive oxygen species; THP, tris(hydroxymethyl)phosphine

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