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# Fluorescent HPLC for the detection of specific protein oxidation carbonyls – α-aminoadipic and γ-glutamic semialdehydes – in meat systems

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ABSTRACT

Precise methodologies for the routine analysis of particular protein carbonyls are required in order to progress in this topic of increasing interest. The present paper originally describes the application of an improved method for the detection of α-aminoadipic and γ-glutamic semialdehydes in a meat system by using a derivatization procedure with p-amino-benzoic acid (ABA) followed by fluorescent high-performance liquid chromatography (HPLC). The method development comprises i) the description of a simple HPLC program which allows the efficient separation of the ABA and the key standard compounds and ii) the optimization of the procedure for the preparation of a meat sample in order to maximize the fluorescent signal for both protein carbonyls. Furthermore, the suitability of this method is evaluated by applying the technique to porcine burger patties. The present procedure enables an accurate and relatively fast analysis of both semialdehydes in meat samples in which they could play an interesting role as reliable indicators of protein oxidation.



## 1. Introduction

The occurrence of protein oxidation has been profusely studied in living systems owing the proved link between the oxidative damage of proteins and the development of age-related diseases (Levine, 2002; Stadtman, 1992). The interest in protein oxidation is sharply increasing among food scientists while the knowledge on this particular topic is still rather limited. Since muscle proteins were found to undergo oxidative reactions and yield protein carbonyls during post-mortem events (Martinaud et al., 1997), numerous works have studied the impact of meat processing and antioxidants on the incidence and extent of protein oxidation (Estévez, Ventanas, & Cava, 2007; Mercier, Gatellier, & Renerre, 1995; Salminen, Estévez, Kivikari, & Heinonen, 2006; Ventanas, Ventanas, Tovar, García, & Estévez, 2007). The oxidation of proteins is a complex phenomenon which has multiple manifestations such as the degradation of amino acid side chains, the formation of cross-links and the breakage of peptide bonds (Stadman & Levine, 2000). The formation of carbonyl compounds from specific amino acids has been highlighted as one of the most remarkable measurable changes in oxidized proteins (Lund, Heinonen, Baron, & Estévez, 2011; Requena, Levine, & Stadtman, 2003). In fact, the quantification of the total amount of protein carbonyls has been commonly used as indicator of protein oxidation in both biological and food systems. A method originally employed in medicine research which involves the derivatization of carbonyl compounds with dinitrophenylhydrazine (DNPH) (Oliver, Ahn, Moerman, Goldstein, & Stadtman, 1987) has been commonly applied for the detection of protein carbonyls in muscle foods. This method, however, is largely criticized by meat scientists due to the difficulties to obtain repeatable and consistent results. Among various drawbacks, the DNPH method would also account lipid-derived carbonyls in muscle foods, miscalculating the total amount of protein carbonyls and hence, leading to unreliable results on the actual extent of protein oxidation (Armenteros, Heinonen, Ollilainen, Toldrá, & Estévez, 2009; Estévez, Morcuende, & Ventanas, 2008). As a poorly specific method, the DNPH procedure provides no information on the chemical structures of the protein carbonyls and the mechanisms involved in the formation of such compounds remain unknown. More specific methodologies are required to fully comprehend the basic chemistry behind the occurrence of protein carbonylation in muscle foods. The identification of particular protein carbonyls would enable, in turn, the accomplishment of challenging studies devoted to shed light on the impact of protein oxidation on muscle foods and the development of more effective antioxidant strategies. Based on a procedure originally conceived for medical research (Akagawa et al., 2006), Estévez, Ollilainen, and Heinonen (2009) recently identified two specific protein carbonyls namely, α-aminoadipic semialdehyde and γ-glutamic semialdehyde (AAS and GGS, respectively), in several oxidized food proteins by using high performance liquid chromatography coupled to electrospray ionization and multi-stage tandem mass spectrometry (LC–ESI–$MS^n$). AAS is the oxidation product of lysine while GGS is originated from the oxidation of arginine and proline (Fig. 1). In a further study (Estévez & Heinonen, 2010), the role of iron and myoglobin as

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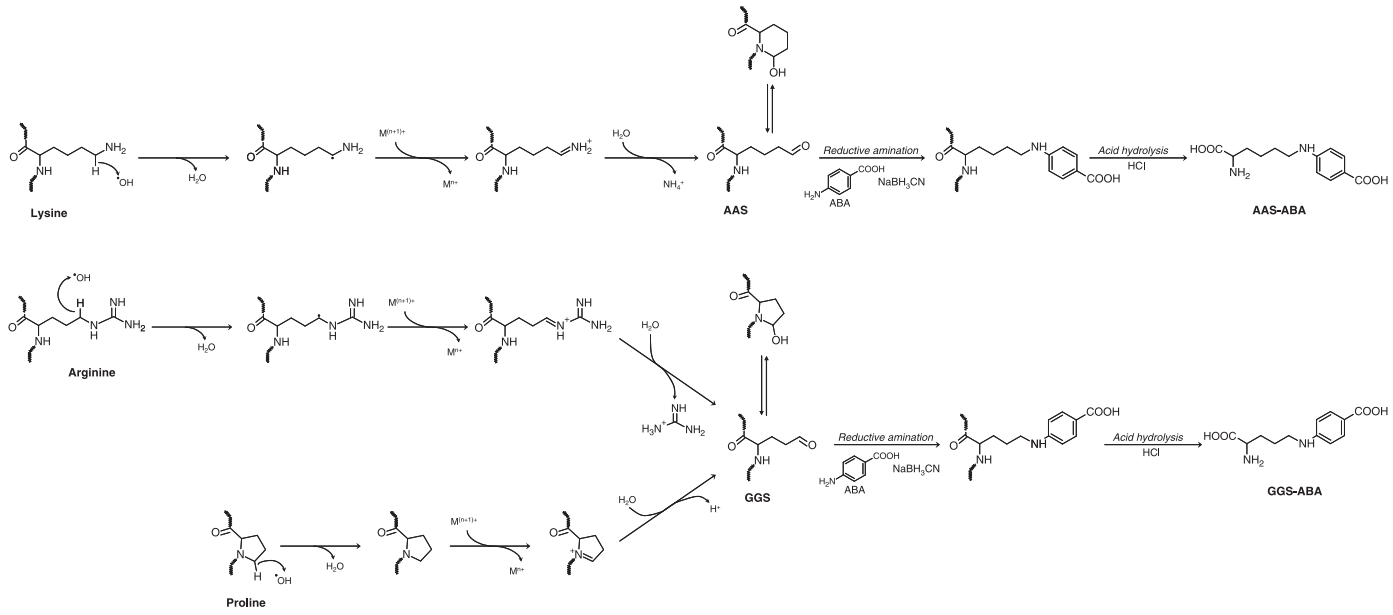


**Fig. 1.** Scheme for the formation of AAS and GGS from lysine and arginine, respectively, and the subsequent derivatization with ABA prior to HPLC-FLD analysis.

promoters of carbonylation in myofibrillar proteins and the complex mechanisms involved in the redox-actions of phenolic compounds was investigated by using this methodology. In due course, the method was applied to several meat products such as raw and cooked patties, cooked sausages and fermented meats (Armenteros et al., 2009; Fuentes, Ventanas, Morcuende, Estévez, & Ventanas, 2010; Ganhão, Morcuende, & Estévez, 2010). The analysis of AAS and GGS, as described in the aforementioned papers, requires costly equipment (LC–ESI–MS) and high-qualified HPLC technicians. Furthermore, the method followed for the preparation of the meat samples, which involves derivatization and hydrolysis procedures, was straightforwardly adapted from medical studies. The suitability of the application of such procedures to meat samples remains unknown. The present paper describes a procedure for the fluorescent detection and quantification of p-amino benzoic acid (ABA)-derivatized forms of AAS and GGS by using an HPLC method. It is also the purpose of the present work to maximize the fluorescent signal of both carbonyl compounds through the optimization of the derivatization and hydrolysis procedures applied to meat samples. The optimized method is then employed for the analysis and quantification of both semialdehydes in burger patties subjected to cooking and chilling procedures.

## 2. Material and methods

### 2.1. Chemicals and raw material

All chemicals and reagents used for the present work were purchased from Panreac (Panreac Química, S. A., Barcelona, Spain), Merck (Merk, Darmstadt, Germany) and Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany). Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA). Porcine meat (muscle *longissimus dorsi*) and back-fat were obtained from a local slaughterhouse.

### 2.2. Synthesis of AAS and GGS standard compounds

N-Acetyl-L-AAS and N-acetyl-L-GGS were synthesized from Nα-acetyl-L-lysine and Nα-acetyl-L-ornithine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa et al. (2006). Briefly, 10 mM Nα-acetyl-L-lysine and Nα-acetyl-L-ornithine were independently incubated with constant stirring with 5 g egg shell membrane in 50 mL of 20 mM sodium phosphate buffer, pH 9.0 at 37 °C for 24 h. The egg shell membrane was then removed by centrifugation and the pH of the solution adjusted to 6.0 using 1 M HCl. The resulting aldehydes were reductively aminated with 3 mmol ABA in the presence of 4.5 mmol sodium cyanoborohydride ($NaBH_3CN$) at 37 °C for 2 h with stirring. Then, ABA derivatives were hydrolyzed by 50 mL of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C *in vacuo* to dryness. The resulting AAS-ABA and GGS-ABA were purified by using silica gel column chromatography and ethyl acetate/acetic acid/water (20:2:1, v/v/v) as elution solvent. The purity of the resulting solution and authenticity of the standard compounds obtained following the aforementioned procedures have been checked by using MS and $^1$H NMR (Akagawa, Suyuma, & Uchida, 2009; Estévez et al., 2009).

### 2.3. Optimization of HPLC separation of standard compounds

The HPLC procedure described by Akagawa et al. (2009) was employed as starting point to optimize the separation of both standard compounds (GGS-ABA and AAS-ABA) and the derivatization agent (ABA) using a COSMOSIL $5C_{18}$-AR-II RP-HPLC column (5 μm, 150 × 4.6 mm) and a guard column (10 × 4.6 mm) filled with the same material. The Shimadzu "Prominence" HPLC apparatus (Shimadzu Corporation, Kyoto, Japan) was equipped with a quaternary solvent delivery system (LC-20AD), DGU-20AS on-line degasser, SIL-20A auto-sampler, RF-10A XL fluorescence detector, and CBM-20A system controller. As a first attempt for the separation of standard compounds, the column was eluted with 50 mM sodium acetate buffer pH 5.4 in accordance to the procedure described by Akagawa et al. (2009) (ISO1). Additional HPLC programs combining two eluents (Eluent A: 50 mM sodium acetate buffer pH 5.4; Eluent B: acetonitrile, ACN) were tested: Isocratic 1% B (ISO2); Isocratic 3% B (ISO3); Isocratic 5% B (ISO4); low pressure gradient by varying B concentration from 0% (min 0) to 8% (min 20) (GRAD). The injection volume of a standard solution mixture containing ABA, AAS-ABA and GGS-ABA was 0.1 μL, the flow rate was kept at 1 mm/min, the temperature of the column was maintained constant at 30 °C and the

length of the run was set at 30 min for all HPLC programs tested except for ISO1 (80 min). The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. The different HPLC programs were compared for their ability to provide a fast elution and efficient separation of the standard compounds.

### 2.4. Manufacture of porcine patties

Porcine patties were manufactured and employed as pertinent meat systems to apply the HPLC-FPD method for the detection of specific protein carbonyls. The ingredients per kg of patty were as follows: 700 g meat (porcine *longissimus dorsi* muscle), 180 g distilled water, 100 g pork back-fat and 20 g sodium chloride. All ingredients were minced in a cutter until a homogeneous raw batter was obtained. Burger patties were formed using a conventional burger-maker (~100 g/patty), to give average dimensions of 10 cm diameter and 1 cm thickness. In total, twelve burger patties were prepared in two independent manufacturing processes (six patties each time). Four patties were frozen (−80 °C) the day of the manufacture and processed for analysis in due course ("Raw patties"), while the other eight were placed on trays and cooked at 170 °C for 18 min in a forced-air oven. After cooking, four samples were allowed to cool down at room temperature and then frozen (−80 °C) until analysis ("Cooked patties") while the other four were dispensed in polypropylene trays wrapped with PVC film and subsequently stored for 15 days at +5 °C in a refrigerator under white fluorescent light (620 lx), simulating retail display conditions ("Cooked and chilled patties"). Upon chill storage, these samples were as well frozen (−80 °C) until required for analysis. All samples were processed for the analyses of AAS and GGS and the total amount of protein carbonyls according to the DNPH method within two weeks following manufacture.

### 2.5. Optimization of meat sample preparation for HPLC-FLD analysis

The derivatization and hydrolysis procedures described by Akagawa et al. (2006) for biological samples were employed as a starting point to optimize the preparation procedure for meat samples. Cooked and chilled patties manufactured as previously described were employed as suitable meat samples. The impact of the initial concentration of muscle protein in the sample (P), the concentration of the derivatization agent (ABA) (D) and the hydrolysis time (T), on the fluorescent signal and hence the calculated amount of the derivatized forms of AAS and GGS in meat samples, was assessed following a Box Behnken design from The Unscrambler® program (v 9.0). According to the model, three levels of the three design variables, namely P (0.01, 0.02 and 0.04 g/mL muscle protein), D (10, 30 and 50 mM ABA) and T (12, 18 and 24 h) were computed to investigate their impact on three response variables, namely the fluorescent signal of ABA, AAS-ABA and GGS-ABA. Solutions with different protein concentration were obtained through the homogenisation of the sample with the appropriate volume of 6 M NaCl 20 mM sodium phosphate buffer pH 6.5 using an ultraturrax homogenizer for 30 s. In accordance to the Box–Behnken design, thirteen randomized samples were prepared in triplicate and treated for reductive amination in the presence of ABA and subsequent hydrolysis according to the procedure described by Akagawa et al. (2006) with the modifications introduced by the present design in terms of ABA concentration and hydrolysis time. A total of HPLC 39 samples were analyzed using the "GRAD" procedure above described and the fluorescent signal of ABA, AAS-ABA and GGS-ABA recorded as response variables. In addition to the effects of the design variables and their interactions on the response variables according to an analysis of variance (ANOVA) with a significance level of 0.05, results from the design were visualized in the form of response surfaces.

### 2.6. Preparation of meat samples for HPLC-FLD analysis

Oxidized proteins from raw (R), cooked (C) and cooked and chilled (CC) burger patties were prepared for HPLC analysis following the method described by Akagawa et al. (2006) with modifications in accordance to the optimization procedure accomplished in the present paper. Burger patties (1 g) were minced and then homogenized 1:10 (w/v) in 6 M NaCl 20 mM sodium phosphate buffer pH 6.5 using an ultraturrax homogenizer for 30 s. Depending on the sample, the final meat homogenates contained from 0.015 to 0.025 g/mL of protein. An aliquot (200 μL) was dispensed in 2 mL screw-capped eppendorf tubes. Proteins were precipitated with 2 mL cold 10% TCA and subsequent centrifugation at 2000 rpm for 30 min. The resulting pellets were treated again with 2 mL cold 5% TCA and proteins precipitated after centrifugation at 5000 rpm for 5 min. Pellets were then treated with 0.5 mL of 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1% sodium dodecyl sulfate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mL of 50 mM ABA in 250 mM MES buffer pH 6.0 and 0.25 mL of 100 mM $NaBH_3CN$ in 250 mM MES buffer pH 6.0. The derivatization was completed by allowing the mixture to react for 90 min while tubes were immersed in a water bath at 37 °C and stirred regularly. All solutions employed for the derivatization procedure were freshly made at sampling days. The derivatization reaction was stopped by adding 0.5 mL of cold 50% TCA followed by a centrifugation at 5000 rpm for 5 min. Pellets were then washed twice with 1 mL of 10% TCA and 1 mL of ethanol-diethyl ether (1:1, v/v). Centrifugations at 5000 rpm for 5 min were performed after each washing step. Protein hydrolysis was performed at 110 °C for 18 h in the presence of 6 M HCl. Hydrolysates were finally dried *in vacuo* at 40 °C using a Savant speed-vac concentrator. Hydrolysates were finally reconstituted with 200 μL Milli-Q water and filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corporation, USA) for HPLC analysis.

### 2.7. HPLC-FLD analysis of meat samples

An aliquot (1 μL) from the reconstituted protein hydrosilates was injected and analyzed in the above mentioned HPLC equipment. The GRAD HPLC program was selected for the separation and subsequent identification of AAS-ABA and GGS-ABA. Standards (0.1 μL) were run and analyzed under the same conditions. Identification of both derivatized semialdehydes in the FLD chromatograms was carried out by comparing their retention times ($R_t$) with those from standard compounds. The peaks corresponding to AAS-ABA and GGS-ABA were manually integrated from FLD chromatograms and the resulting areas plotted against an ABA standard curve with known concentrations that ranged from 0.1 to 0.5 mM. Regression coefficients greater than 0.99 were obtained. The estimation of the quantities of AAS-ABA and GGS-ABA through an ABA standard curve was accomplished by assuming that the fluorescence emitted by 1 mol of ABA is equivalent to that emitted by 1 mol of derivatized protein carbonyl. Results are expressed as nmol of carbonyl compound per mg of protein. Protein was quantified in burger patties by the official AOAC method (AOAC, 2000).

### 2.8. Determination of total carbonyls by the DNPH-method

Total protein carbonyls were quantified in raw, cooked and cooked and chilled burger patties according to the method described by Oliver et al. (1987) with minor modifications. Samples were homogenized (1:10 w/v) in 0.6 N NaCl 20 mM sodium phosphate buffer, pH 6.5 and an aliquot (100 μL) of meat homogenate was dispensed in 2 mL eppendorf tubes. Proteins were precipitated by cold 10% TCA (1 mL) and subsequent centrifugation for 5 min at 5000 rpm. One pellet was treated with 1 mL 2 M HCl (protein concentration

measurement) and the other with an equal volume of 0.2% (w/v) DNPH in 2 M HCl (carbonyl concentration measurement). Both samples were incubated for 1 h at room temperature. Afterwards, samples were precipitated by 10% TCA (0.8 mL) and washed twice with 1 mL ethanol:ethyl acetate (1:1, v/v) to remove excess of DNPH. The pellets were then dissolved in 2 mL of 6 M guanidine HCl in 20 mM sodium phosphate buffer pH 6.5, stirred and centrifuged for 2 min at 5000 rpm to remove insoluble fragments. Protein concentration was calculated from absorption at 280 nm using BSA as standard. The amount of carbonyls was expressed as nmol of carbonyl per mg of protein using an absorption coefficient of 21.0 $nM^{-1}cm^{-1}$ at 370 nm for protein hydrazones.

### 2.9. Statistical analysis

In order to compare between types of patties (Raw, Cooked and Cooked and Chilled) for the amount of protein carbonyls, an Analysis of Variance (SPSS, v. 15) was accomplished. The statistical significance was set at $p<0.05$.

## 3. Results and discussion

### 3.1. Detection and separation of standard compounds by HPLC-FLD

Major protein carbonyls, AAS and GGS, are highly labile in the presence of strong acids. The reductive amination with ABA and $NaBH_3CN$ provides stability to the derivatives against acid hydrolysis and enables their detection by using fluorescent HPLC (Akagawa et al., 2006, 2009). The fluorescence emitted by the derivatization agent (ABA), added in excess, may interfere with the fluorescent signal of the derivatized forms of AAS and GGS and hence, it is essential to assure an efficient separation of the three compounds. In the present study, the application of the HPLC program (ISO1) described by Akagawa et al. (2009) allowed the detection of the three peaks of interest namely, ABA ($R_t=8.8$), GGS–ABA ($R_t=15.5$) and AAS–ABA ($R_t=49.7$) (Fig. 2a). However, the time required for the elution of the three compounds was considerably larger than the time reported by Akagawa et al. (2009) (50 min *vs* 20 min). These authors employed a greater flow rate (1.5 mL/min) and a longer version (5 μ, 4.6×250 mm) of the column used in the present study. In order to shorten the run time, increasing percents of ACN (1, 3 and 5%) were mixed to the 50 mM acetate buffer pH 5.4 to create the corresponding isocratic HPLC programs (ISO 2, 3 and 4 respectively; Fig. 2). As expected, the ACN accelerated the elution of the standard compounds and improved the efficacy of the corresponding peaks. However, the reduction of the time elapsed between the eluted peaks led, in some cases, to an inefficient separation between ABA and GGS–ABA (Figs. 2c and d). A simple and slight gradient of ACN from min 0 (0% ACN) to min 20 (8% ACN) enabled a clear resolution of the three compounds which eluted within the first 20 min of the run ($R_t$ ABA = 8.1; $R_t$ GGS-ABA = 10.7; $R_t$ AAS-ABA = 16.9) (Fig. 2e). Compared to the HPLC program reported by Akagawa et al. (2009), the GRAD program allows a faster elution and more efficient separation of the three compounds. Following the procedure reported by the aforementioned authors, the

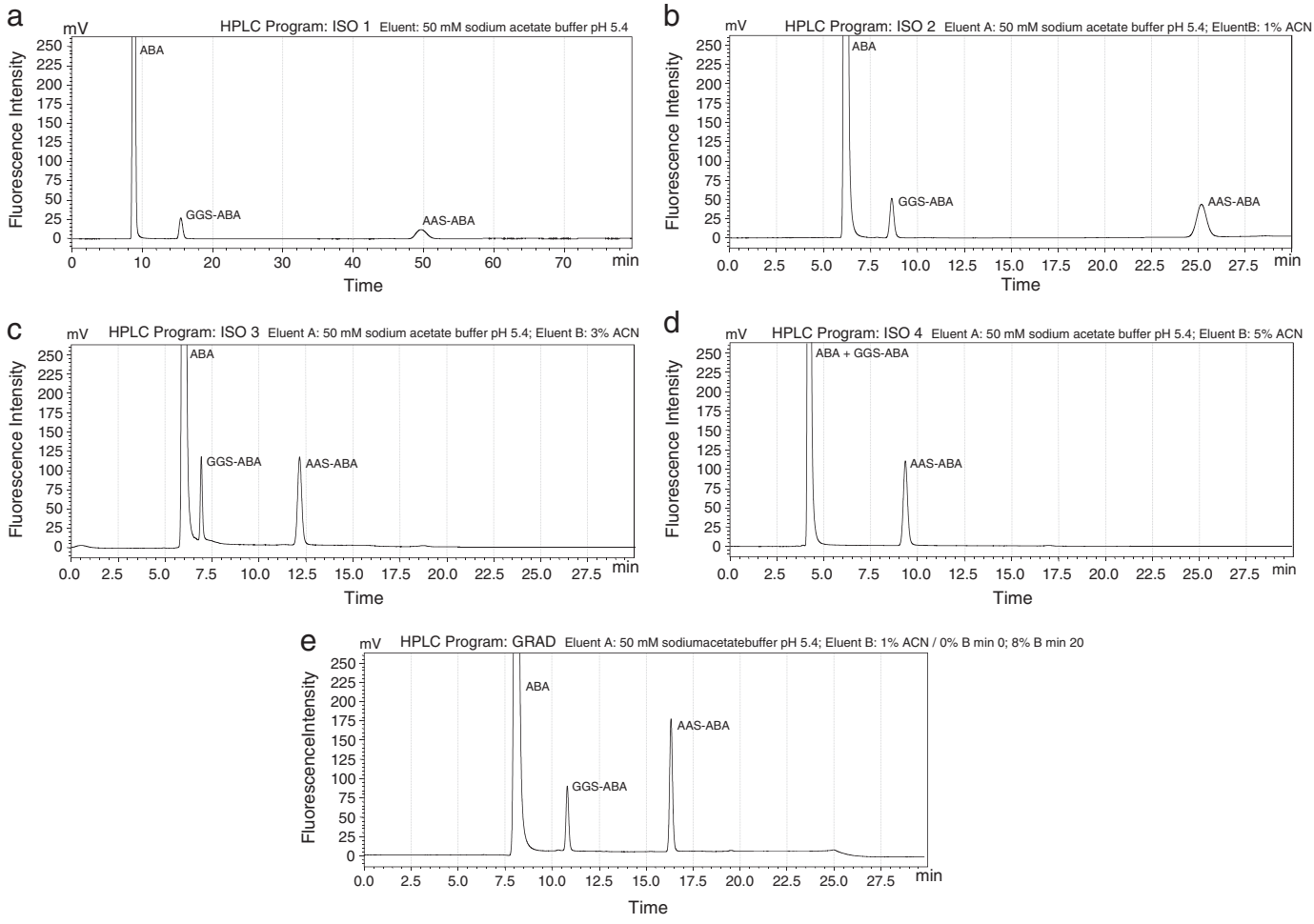


**Fig. 2.** FLD chromatograms of a standard solution containing ABA, GGS and AAS obtained upon the application of different HPLC programs.

first standard (GGS–ABA) elutes in the tail of the ABA peak, which may interfere with a correct integration of the peak of interest. The method (GRAD) proposed in the present paper enables short runs as the initial conditions, including initial pressure and stable baseline, are re-established in just 30 min. By using a shorter version of the column employed by Akagawa et al. (2009) and a lower flow rate, the total volume of eluent required per run is considerably reduced. According to our calculations, the detection and quantification limits for the analyzed compounds were approximately 1.5 and 5 fmol, with signal-to-noise ratios of 3:1 and 10:1, respectively. The interday and intraday repeatability of the HPLC analysis was satisfactory (less than 3%).

### 3.2. Optimization of meat sample preparation for HPLC-FLD analysis

The HPLC analysis of AAS and GGS subsequent to the derivatization with ABA is a procedure originally conceived for plasma proteins and biological systems (Akagawa et al., 2006). As usual for methods aimed to assess oxidative stress, the above procedure was consequently applied to food systems (Estévez et al., 2009). The rational adaptation of the method to meat systems should involve a previous study to guarantee that the original derivatization and hydrolysis procedures are suitable for muscle proteins. In this sense, a meat sample was processed in the present study for the amount of AAS and GGS by using several protein concentrations, ABA concentrations and hydrolysis times. As expected, the concentration of muscle protein significantly influenced the amount of AAS and GGS (Table 1). The fluorescent signal for both carbonyl compounds enlarged with increasing amounts of muscle protein and such relationship was proportional. This likely finding remarks the reliability between the amount of protein carbonyls and the concentration of protein in the sample and the importance of expressing the results as protein units. Akagawa et al. (2006) reported that at least 5 mg of plasma proteins are required to determine AAS and GGS in biological systems. The present study shows that both semialdehydes can be detected in 2 mg of muscle protein from a meat product such as a cooked patty. A protein concentration within the range assessed in the present work can be easily achieved for any muscle food by performing the correct dilution in the phosphate buffer prior to homogenisation. Surprisingly, the concentration of protein affects positively to the fluorescent signal of ABA which could be explained by the difficulty to remove the excess of the derivatization agent from meat samples with high protein levels. Fig. 3 shows the response surfaces corresponding to the effects of the hydrolysis time and the concentration of ABA on the fluorescent signal of AAS–ABA and GGS–ABA analyzed from an intermediate concentration of muscle protein (0.02 g/mL). The hydrolysis time had no effect on the fluorescent signal of GGS-ABA but influenced significantly the signal of AAS-ABA. These results suggest that 12 h of acid hydrolysis would be enough to release and detect the total amount of derivatized GGS from the meat sample. On the other hand, 18 h of acid hydrolysis is required to obtain the largest fluorescent signal for AAS-ABA. According to the present results, the original procedure could be shortened from 24 to 18 h to obtain the maximum fluorescent signal from both compounds. The concentration of the derivatization agent in the studied range (10–50 mM) does not affect the fluorescent detection of AAS while the detection of the total amount of GGS requires using the largest concentration of ABA (50 mM). Using low concentrations of the derivatization agent is commonly appreciated owing the cost of chemicals, the possible interferences caused by the fluorescent signal from the excess of ABA, and the impact of that on the HPLC equipment. Using 10 mM may be enough to analyze the total amount of AAS from a moderately oxidized meat sample containing around 0.02 g/mL muscle protein while a larger ABA concentration (50 mM) may be required for the detection of the total amount of GGS.

**Table 1**
Effects of "Protein Concentration", "ABA concentration" and "Hydrolysis Time" and the corresponding interactions on the amount of ABA, AAS and GGS in porcine patties.

| Variable | ABA | AAS | GGS |
|---|---|---|---|
| Derivatization agent concentration (A) | +++ | NS | + |
| Hydrolysis time (B) | NS | + | NS |
| Protein concentration (C) | + | +++ | +++ |
| A×B | NS | NS | NS |
| A×C | NS | NS | NS |
| B×C | NS | NS | NS |

+: $p<0.05$.
+++: $p<0.001$.
NS: no significant.
A×B, A×C and B×C: corresponding interactions between the effects, A, B and C.

### 3.3. Application of the HPLC-FLD method in burger patties

In order to confirm that the improvements and modifications derived from the previous experiments are suitable for meat products, the GRAD HPLC program was applied to raw, cooked and cooked and chilled burger patties. To fulfill with objective, homogenates with approximately 0.02 g/mL of protein were prepared from meat samples. In order to assure the maximum fluorescent signal from both compounds, the meat samples were derivatized in the presence of 50 mM ABA and hydrolyzed for 18 h. Both semialdehydes were efficiently detected in all meat samples and eluted in accordance to the results previously found for standard compounds. Table 2 shows the amounts of both semialdehydes as well as the total amount of protein carbonyls by the DNPH method. As expected, raw patties contained low amounts of both semialdehydes. The formation of AAS and GGS is known to take place *in vivo* as a result of the oxidative stress of proteins in animal and human tissues. According to Akagawa et al. (2006) rat liver proteins contain around 0.05 mmol AAS/mg protein and 0.04 mmol GGS/mg protein while human plasma proteins have 0.02 mmol AAS/mg protein and 0.01 mmol GGS/mg protein. Similar results were obtained for Daneshvar, Frandsen, Autrup, and Dragsted (1997) in animal tissues. The present results suggest that post-mortem events and certain processing technologies (mincing, chilling…) could have promoted the formation of both semialdehydes up to the levels found in raw patties. Taking into consideration the well-established role of iron and myoglobin on the formation of carbonyl compounds from myofibrillar proteins (Estévez & Heinonen, 2010), it is plausible that these oxidation promoters would initiate the oxidative degradation of susceptible amino acids in meat systems upon slaughter and during meat handling, processing and storage. The amount of both semialdehydes significantly increased as a result of cooking, highlighting the impact of high temperatures on the oxidative stability of susceptible amino acids. The high temperatures reached during meat cooking enhance the formation of ROS and hence, the oxidative degradation of lipids and proteins (Ganhão et al., 2010; Kanner, 1994). According to the present results, muscle proteins from cooked patties underwent further oxidative reactions during the subsequent refrigerated storage. Cooked and chilled burger patties had the largest amount of both semialdehydes. The acceleration of oxidative reactions in cooked meats has been previously described for lipid and protein oxidation (Estévez, Ventanas, & Cava, 2004; Ganhão et al., 2010) and might be caused by a number of factors including the disruption of cellular compartmentalization and exposure of membrane lipids to a pro-oxidative environment and release of catalytic free iron from myoglobin, among others. The amounts of AAS and GGS found in cooked and chilled patties are comparable to those found in various plasma proteins oxidized *in vitro* (Akagawa et al., 2006; Daneshvar et al., 1997; Requena, Chao, Levine, & Stadtman, 2001). As usually reported for various mammalian proteins (Akagawa et al., 2006; Requena et al., 2001), AAS is found in

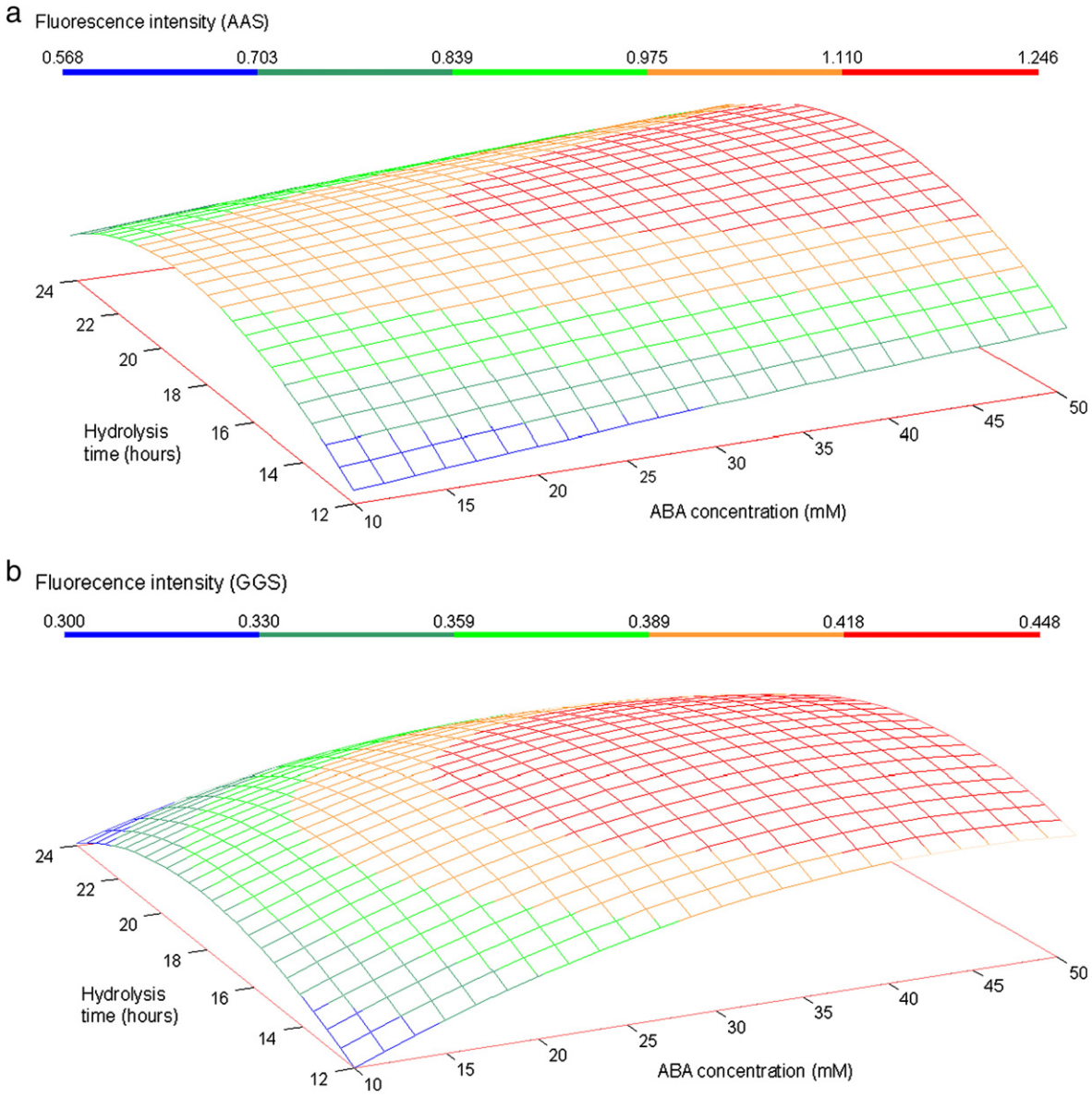


**Fig. 3.** Response surface corresponding to the effects "ABA concentration" and "Hydrolysis Time" on the amount of AAS (a) and GGS (b).

larger quantities than GGS in meat systems. The results from the present study are in agreement with those recently reported by Ganhão et al. (2010) in porcine burger patties. In that previous paper, the mechanisms involved in the oxidative deamination of lysine, proline and arginine for the formation of AAS and GGS during cooking and chilling of meat patties were profusely described. Unlike that study, the present paper originally provides quantitative results on the formation of specific protein carbonyls in a meat product. The total amount of protein carbonyls as quantified by using the DNPH method was noticeably higher than the sum of AAS and GGS. Both semialdehydes accounted for 30–60% of the total DNPH-carbonyls. These results are in good agreement with previous reports by Requena et al. (2001) and Akagawa et al. (2006) who reported that AAS and GGS constituted between 23% and 60% of the total protein carbonyls of oxidized liver and plasma proteins. Amino acid residues other than lysine, proline and arginine could yield carbonyl compounds upon oxidation (Levine, 2002; Lund et al., 2011) and hence, partly explain the disparity of quantities between methods. However, this lack of consistency may be also explained by the lack of specificity of the spectrophotometric method as the DNPH is attached to lipid-derived carbonyls such as malondialdehyde leading to the overestimation the total amount of protein carbonyls. The DNPH method has been criticized for this drawback in a previous paper

**Table 2**
Amounts[a] of protein carbonyls (means ± standard deviation) in raw, cooked and cooked and chilled patties.

| | Raw patty | Cooked patty | Cooked and chilled patty | p-value |
|---|---|---|---|---|
| GGS | $0.13^{b} \pm 0.04$ | $0.38^{c} \pm 0.10$ | $0.59^{a} \pm 0.10$ | <0.001 |
| AAS | $0.27^{b} \pm 0.04$ | $0.76^{c} \pm 0.16$ | $1.09^{a} \pm 0.24$ | <0.001 |
| Total carbonyls[c] | $0.40^{b} \pm 0.07$ | $1.14^{c} \pm 0.20$ | $1.68^{a} \pm 0.22$ | <0.001 |
| Total carbonyls[b] | $1.31^{b} \pm 0.34$ | $1.87^{c} \pm 0.32$ | $3.42^{a} \pm 0.66$ | <0.001 |

Means within the same line with different superscript were significantly different ($p<0.05$).
[a] Expressed as nmol/mg protein.
[b] Total amount of protein hydrazones as quantified by the DNPH method.
[c] Sum of GGS and AAS.

(Armenteros et al., 2009) and various review articles (Estévez et al., 2009; Lund et al., 2011).

## 4. Conclusions

Unlike the DNPH method, the quantification of AAS and GGS by using HPLC-FLD enables a specific and accurate assessment of protein carbonylation in meat products. The analysis of particular protein carbonyls by using the present methodology allows the identification of the chemical nature of the compounds and the comprehension of the oxidation pathways and the involved mechanisms. The present paper provides a simple and relatively fast procedure for the reliable quantification of AAS and GGS in meat products. The application of this method in future studies will boost the progress in this field of increasing interest among meat researchers.

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