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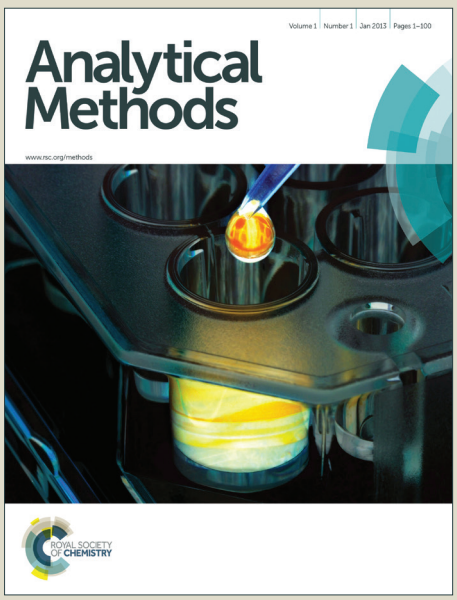

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DEVELOPMENT OF AN ANALYTICAL METHOD TO DETERMINE MALONDIALDEHYDE AS AN OXIDATIVE MARKER IN CRYOPRESERVED BOVINE SEMEN

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Abstract

Frozen sperm is widely used in artificial insemination of cattle as well as other animal species. As a consequence of the freezing and thawing processes of semen, an excess of reactive oxygen species (ROS) are formed. ROS produce oxidative damage in sperm cells affecting both motility and fertility. Malondialdehyde (MDA) is one of the most recognized biomarkers of the advanced oxidative status. MDA was analyzed after its condensation reaction with thiobarbituric acid (TBA); however, other molecules can also react with TBA. In order to determine specifically MDA-TBA_2 condensation product in cryopreserved bovine semen, a sensitive, and selective separative strategy was developed using high performance liquid chromatography (HPLC) with diode array detection (DAD). This is the first report of MDA determination in bovine semen by a separative method. Different methodological approaches were assayed. Treatment A directly measured total MDA through acidic hydrolysis combined with TBA condensation in a single step. Treatment B evaluated separately TBA condensation product of free MDA and protein bound MDA after its releasing with alkaline hydrolysis. The highest concentration of MDA was detected following treatment A. An HPLC method was developed and validated comparing with the traditional spectrophotometric method. The detection and quantification limits were 0.034 μM and 0.086 μM. The DAD response was linear in the range between 0.086 and 9.1 μM. The recovery was 91%. The intra and interday relative standard deviations were 3.7 and 3.8%, respectively. The HPLC proposed method was markedly more sensitive and more specific than the traditional spectrophotometric one.

Keywords: Lipid Oxidation – Spermatozoa – TBARS – HPLC – MDA

Abbreviations:

ACN: acetonitrile; DAD: diode array detection; HPLC: high performance liquid chromatography; LOD: limit of detection; LOQ: limit of quantification; LPO: lipid peroxidation; MDA: malondialdehyde; PB: protein bound; ROS: reactive oxygen species; RSD: relative standard deviation; TBA: thiobarbituric acid; TBARS: thiobarbituric acid reactive species; TCA: trichloroacetic acid; TEP: 1,1,3,3- tetraetoxypropane.

1. Introduction

Frozen sperm is widely used in artificial insemination of cattle as well as other animal species. It has been found that the number of apoptotic cells in the cryopreserved material is larger than that of fresh semen, independently of freezing and thawing techniques used.[1] Semen storage, particularly in frozen state, causes biochemical and functional changes in sperm cells affecting their motility and fertility. Low temperatures produce damages in the plasmatic and acrosome membranes, in the mitochondria and in the axonema sheath of spermatic cell. Damaged cells produce a large number of reactive oxygen species (ROS). This excess of ROS has a negative impact on the rest of viable cells.[2] However, ROS have a key role in the normal sperm physiology because they are formed during mitochondrial respiration and are involved in the maintenance of the fertilizing ability and the capacitation/ acrosome reaction of spermatozoa.[3]

In order to prevent ROS adverse effects, biological systems possess their own antioxidant mechanisms involving enzyme systems and various organic molecules, such as vitamins E and C.[4] This antioxidant complex can be formed by glutathione peroxidase, catalase, and superoxide dismutase, among other enzymes. This enzymatic system has also been found to prevent oxidative damage in spermatozoa and seminal plasma, and, therefore, to maintain sperm motility and viability.[5]

Nevertheless, the imbalance between ROS production and removal, called oxidative stress, causes adverse effects on spermatozoa. ROS may attack target molecules such as lipids, DNA, and proteins. Taking into account that sperm cells contain high concentrations of polyunsaturated fatty acids, they are highly susceptible to ROS attack, leading to the process known as lipid peroxidation

(LPO).[6] This oxidation leads to subsequent losses of motility, membrane integrity, and fertilizing capability as well as metabolic changes of sperm .[5] LPO is a radical chain reaction whose primary oxidation products are lipid hydroperoxides. These compounds are unstable and prone to degradation; thus, the oxidative process continues forming diverse secondary oxidation products like aldehydes, ketones, and short-chain carboxylic acids.[4] Hydroperoxides quantification is not convenient due their intrinsic instability. Hence, LPO's progress is better determined by quantifying some secondary product levels. Among the final oxidation products, malondialdehyde (MDA) is one of the most recognized biomarkers of an advanced oxidative status.[7] MDA measurement is considered an objective parameter of spermatozoa quality, and its determination has been previously reported in sperm cells and seminal plasma of various species.[1-3] In this sense, a correlation between the increase of MDA level in semen samples and the decrease of sperm cell motility has been demonstrated.[8]

Several strategies leading to the derivatization of the carbonyl functional groups of MDA have been proposed using reagents such as hydrazine-based agents: dinitrophenylhydrazine (DNPH)[9], FMOC-hydrazine[10] or hydralazine.[11] One of the most serious drawbacks related to hydrazines is that this method requires a time-consuming step as sample pre-treatment with multiple reactions.

Another alternative on MDA derivatization and its subsequent measurement is the condensation of two molecules of thiobarbituric acid (TBA) with MDA.[7] This assay has been widely used to assess lipid oxidative damage in various biological systems as an effective quantitative assay. Thus, the MDA-TBA_2 condensation product is usually determined by UV-Vis

spectrophotometry as well as by spectrofluorometry.[8] TBA assay is known as thiobarbituric acid reactive species (TBARS) test because TBA may also react with a variety of compounds, such as sugars, amino acids, a variety of aldehydes and bilirubin, producing interferences with spectrophotometric and fluorometric measurements of MDA.[12] Besides, the poor specificity associated with TBARS test may lead to an overestimation of the levels of MDA in human plasma and other biological fluids. This may limit the possibility of detecting true differences in the level of lipid peroxidation in clinical studies.[13] Even so, TBARS assay still remains being reported as the main method to determine MDA formation in biological systems. This analytical limitation may be effectively solved by using a separative method to evaluate specifically the MDA-TBA_2 adduct. In this sense, several high performance liquid chromatography (HPLC) methods have been reported for the determination of MDA in human plasma with colorimetric and fluorometric detection, avoiding thus matrix interferences.[14] Additionally, liquid chromatography coupled with mass spectrometry[15] as well as capillary electrophoresis,[16] have also been used for MDA analysis. Among this methods, HPLC remains as the most common method for the determination of MDA because of its simplicity and also good selectivity and sensitivity.

In biological systems, lipids and their oxidation products usually are able to associate with non-lipid compounds (proteins, carbohydrates and water). Thus, MDA can be linked to SH and NH_2 groups of proteins and nucleic acids. Free MDA may directly be measured by TBA reaction; although, to determine protein bound (PB) MDA, an alkaline hydrolysis step previous to condensation reaction is required as reported in plasma samples. [13,17] However, some authors have

previously reported the use of the acidic hydrolysis in semen samples to evaluate MDA total concentration by spectrophotometry.[18]

The main aim of this work was to optimize MDA determination in cryopreserved bovine semen after its condensation with TBA. Different sample pre-treatments were contrasted in order to find the most appropriate one for total MDA measurement: A) via acidic hydrolysis and B) considering both free MDA and PB MDA through alkaline hydrolysis. Secondly, the best pre-treatment selected was assayed to quantify MDA by HPLC with diode array detection (DAD) and compared to the traditional spectrophotometric method. Finally, the HPLC proposed method was validated following the International Conference Harmonization Q2 (R1) guidance.[19] To the best of our knowledge, this is the first report concerning the HPLC analysis of MDA in cryopreserved bovine semen.

2. Experimental Section

2.1. Chemicals and reagents

As precursor of MDA, 1,1,3,3-tetraethoxypropane (TEP) or malondialdehyde *bis*-(diethyl acetal) was purchased from Sigma (Buenos Aires, Argentina). TBA was purchased from Merck (Buenos Aires, Argentina). Chromatography-grade acetic acid and acetonitrile were obtained from Sintorgan (Buenos Aires, Argentina). Milli-Q Water was used to prepare all the aqueous solutions.

2.2. Semen samples

Cryopreserved semen was purchased from an insemination artificial center (CIAVT, Santa Fe, Argentina). Sperm samples proceeding from the same ejaculate of a Bradford bull contained in

0.5 mL straws were frozen and stored in liquid nitrogen until analysis. Each straw had a total of 20.5 million sperm cells equivalent to a concentration of 41 million of sperm cells mL^{-1}.

2.3. Preparation of MDA stock solution and standard solutions

A TEP stock solution in 50:50 (v/v) methanol-water was prepared to a concentration of 1.1 mM. Finally, TEP standard solutions for calibration purposes were prepared in a 1% v/v H_2SO_4 solution with subsequent incubation to hydrolyze TEP into MDA at 40 ºC for 30 min. The final concentration of MDA in every standard solution was determined by measuring its absorbance at 245 nm (ε = 13,700 M^{-1} cm^{-1}).[20]

2.4. Instrumentation and conditions

HPLC analyses were carried out with a Lab Alliance Series III-5 mL chromatographic system, equipped with two pumps and a column thermostating oven. A Grace Vydac Protein & Peptide C_{18} column (250 x 4.6 mm ID, 5 µm) was used. Injection volume was 20 µL. Mobile phase elution was carried out at 1 mL min^{-1} using a binary system; solvent A being a 95:5 (v/v) mixture of 0.57 M (pH 2.5) acetic acid solution and ACN and solvent B being pure ACN. Gradient started with 100% solvent A for 2 min, increased to 20% solvent B at 3.0 min, held for 8.0 min and returned to the initial conditions for 4.0 min. Thus, the total chromatographic run time was 15 min. The column was thermostatized at 40 ºC. The detection wavelength was fixed at 532 nm using a Shimadzu photodiode array detector.

Spectrophotometric analyses were carried out in Unicam UV2 equipment. Absorption spectra were scanned between 200 and 700 nm. Adduct absorbance was measured at 532 nm discounting background absorbance at 700 nm as scattered light.

2.5. Sample preparation

Cryopreserved semen samples were conditioned for analysis as previously reported with minor changes.[21] Straws were thawed in a water bath at 37 ºC for 30 s. Samples were centrifuged at 800×*g* at 25 °C for 15 min. The sperm pellet resulting from the seminal plasma and diluent separation from the cryopreserved semen was re-dissolved in a 1 mL NaH_2PO_4 buffer solution (0.050 M, pH 7.0) to constitute a sample with a sperm concentration of 20.5 million cells mL^{-1}. A general scheme of the procedure was outlined in Fig. 1.

MDA total levels were determined according to two strategies as follows: (A) Total MDA after acidic hydrolysis and (B) as the sum of free MDA and PB MDA, the latter obtained by alkaline hydrolysis procedure.

(A) Total MDA quantification. MDA level was determined as the TBA complex after acidic hydrolysis in one-step reaction as suggested by Suleiman *et al.*[18], with minor modifications. A 2.0 mL aliquot of 0.5% w/v TBA in 20% w/v trichloroacetic acid (TCA) solution was added to 1 mL of the sperm emulsion of 20.5 million cells mL^{-1}. The mixture at pH 2.5 was diluted up to 5 mL with Milli-Q water and was incubated in a vaseline bath at 100 ºC for 15 min. Afterwards, it was cooled in ice bath for 10 min. Then, the emulsion was centrifuged at 10,000 rpm at 4 ºC for 20 min. The supernatant was separated and used for spectrophotometric and HPLC analyses.

(B) Free and PB MDA**.** To estimate MDA distributed in both fractions, Pilz *et al.*[17] and Roca *et al*. [20] procedures were combined with some minor modifications. A 1 mL portion of sperm emulsion of 20.5 million cells mL^{-1} was mixed with 0.5 mL 20% (w/v) TCA in order to precipitate proteins. Sample was centrifuged at 10,000 rpm for 20 min at 4 ºC to separate free MDA

in supernatant and PB MDA in pellet. Free MDA was determined by addition of 2.0 mL 0.5% (w/v) TBA solution in 20% (w/v) TCA and taken to 5 mL. The dilution was incubated at 100 ºC for 15 min. Subsequently, the mixture was cooled in ice bath for 10 min, and finally, the emulsion was centrifuged at 10,000 rpm at 4 ºC for 20 min. The supernatant was then separated for spectrophotometric and HPLC analysis. PB MDA was recovered from the pellet. The precipitate was re-dissolved in 0.25 mL 3 N NaOH. This mixture being at pH 12 was placed into a water bath at 60 ºC for 45 min to hydrolyze MDA-proteins bindings. An aliquot of 0.75 mL of 6% v/v H_3PO_4 and 2.0 mL of 0.5% w/v TBA in 20% w/v TCA were added to this sample. This mixture being at pH 2.5 was taken to 5 mL and kept at 100 ºC for 15 min. After cooling, the sample was centrifuged and supernatant was analyzed by UV-Vis spectrophotometry and HPLC.

2.6. Validation and Analytical Parameters

Once determined the most appropriate option for the sample pre-treatment, the method was validated by measuring the analytical parameters of sensitivity, linearity, precision, and accuracy. Sensitivity was evaluated by determining the limits of detection (LOD) and quantification (LOQ), and basing their calculations on the ratios of 3 and 10 times between the standard deviation of the blank response and the slope of the calibration curve, as defined by International Conference Harmonisation.[19] Linear range was established using at least seven MDA concentrations within the working range. Standard solutions were prepared in triplicate. Precision was estimated as the relative standard deviation (RSD) considering both, intraday and interday precision. Method accuracy was determined by the addition of known amounts of standard MDA to a homogeneous sample to achieve different concentrations within a linear range (0.34; 0.68; and 1.8 μM). The first

addition was equivalent to MDA basal level in order to duplicate the initial level of 0.30 μM; while for the third addition the increase factor is about 6 times the basal level. Spiked samples were prepared with three replicates for each MDA addition. Accuracy was reported as the recovery percentage. Samples were also processed in three different days as described above to establish the interday precision of the validated method. Besides, the long-term stability of the MDA-TBA_2 condensation product was monitored to evaluate the possibility of delaying the analysis once the samples were processed. For this, spiked samples corresponding to 0.34 μM MDA addition were stored at three different temperatures (25, 8, and -20 ºC), and were analyzed along twenty days by the HPLC proposed method as well as the traditional spectrophotometric one.

2.7. Statistical Analyses

The results were performed using the *t*-test for a mean and analysis of variance (ANOVA) statistical package of INFOSTAT version 2012. InfoStat Group, Faculty of Agricultural Sciences, National University of Cordoba, Argentina. Fischer LSD-test was used to compare means when the effects were found to be significant ($P < 0.05$).

2.8. Live subject statement

Authors declare that all experiments were performed in compliance with the relevant laws and institutional guidelines (CONICET, INTA and National University of Santiago del Estero regulations) that have approved the experiments. Authors have not been involved in sample extraction; besides, they have not been in contact with the animal nor handling them.

3. Results and Discussion

3.1 Optimization of the chromatographic conditions

Several chromatographic conditions were assayed for the analysis of MDA-TBA_2 condensation product by changing the mobile phase composition and gradient program in reverse phase conditions (data not shown). The most convenient chromatographic conditions were selected considering the shortest run time, the best peak shape and an adequate retention time for the analyte. The mobile phase consisted of a mixture of acetic acid aqueous solution and ACN, without exceeding 24% in the mixture proportion. The MDA-TBA_2 retention time was 6.3 min, the total chromatographic run time being 15 min. Under these conditions, sample contamination or sample to sample carryover was not observed. To check this, solvent as blank solution was injected every ten sample runs and the peak area corresponding to the target compound was under the detection limit. This short run time allows us to analyze a large number of samples per day. The use of this experimental method in the analysis of cryopreserved bovine semen samples was satisfactory. Representative chromatograms of these biological samples with and without MDA standard addition are shown in Fig. 2. Optimal conditions of chromatographic analyzes were reached without using buffer as mobile phase; in contrast to what is suggested in the literature. High concentrations of phosphate salts, such as the normally used 50 mM, can precipitate inside the analytic column, increasing system pressure and damaging the chromatographic system.[14] As buffer was not needed, this drawback was overcome.

Temperature was an important optimization factor because peak distortions were initially observed and afterwards corrected by thermostating the analytical column (Fig. 3). Preliminary assays were carried out at room temperature as suggested by Karatas.[22] Nonetheless, anomalies such as wide and double elution peaks were observed. These might be attributed to the poor

resolution of the *cis* and *trans* isomers of the MDA-TBA_2 condensation product at room temperature. Davey[23] reported that this problem could be solved by increasing the proportion of organic solvent in the mobile phase. In our experiments, changes of mobile phase composition did not improve the chromatographic peak resolution; therefore, different column thermostating temperatures were assayed in this work. It was found that these anomalities were reduced at 40 °C and satisfactory analytical conditions were reached, coinciding with Grotto statements.[14] In addition, the overall run time was reduced from 30 to 15 min and the time required for column stabilization was also minimized without affecting quantification process.

Furthermore, the ACN proportion in the mobile phase was lower than those proposed in previous reports.[14,17] Indeed, the problem related to ACN scarcity is globally known; in addition to the modern trends to reduce the solvent consumption as an important aim to achieve eco-friendly methodologies.

Besides at different pH of the mobile phase, statistically significant differences were also observed in areas ($P = 0.0001$) of MDA-TBA_2 condensation product. The quantified area for the analyte at pH 2.5 was about the double of the area at pH 3.0. This result can be explained considering that higher acid concentration favors the formation of the MDA-TBA_2 condensation product.[7]

3.2 MDA Determination after Treatments A and B

MDA concentrations were determined by the optimized HPLC method comparing with the traditional spectrophotometric one. Table 1 shows the results according the treatments A and B above mentioned. Thus, in the case of the procedure A, total MDA concentration was determined in

a single step through acidic hydrolytic treatment combined with TBA condensation reaction. Meanwhile, for the strategy B, total MDA concentration was equal to the sum of free MDA and PB MDA levels. In the procedure B, the optimum hydrolysis time to released MDA from binding proteins was 45 min (Fig. A in Electronic Supplementary Data). Mean MDA concentrations found according to procedure A and B were statistically different (P = 0.0001 and P = 0.0021). The highest MDA concentration was measured following the treatment A, in contrast to what takes place in bovine plasma samples. This result can be explained considering that in semen samples the MDA-protein binding is not as strong as in plasma samples.[24] Then, MDA can be released with just an acidic hydrolytic treatment combined with TBA condensation in one step, being unnecessary the alkaline hydrolysis, and hence, reducing the sample preparation time. In addition, results of accuracy assays with the three levels of MDA addition showed greater MDA average recoveries for the pre-treatment A than for treatment B (91% against 81%, respectively, in HPLC analysis; while, in spectrophotometric analysis, 90% against 80%). This result evidences and demonstrates the importance of the present work since the most convenient methodology to analyze MDA differs among different biological fluids according to their chemical composition.

3.3 Validation and Analytical Parameters

After establishing the treatment A as the most appropriate sample pre-treatment and the optimum chromatographic conditions, the HPLC method to determine MDA concentration was validated using MDA quantitatively released from TEP, as validation standard. Thus, the different analytical parameters were determined for both, the HPLC proposed method and the traditional spectrophotometric method.

In this sense, sensitivity corresponds to the minimum analyte amount that can produce a meaningful result; and linearity may be defined as the ability of an analytical procedure to obtain results directly proportional between analyte concentration and its response or analytical signal within a given range. As Table 2 shows, the HPLC method was markedly more sensitive than the spectrophotometric one because in the former method, the detection and quantification limits were the lowest. Fig. 4 corroborates this assessment. The LOD and LOQ of the HPLC proposed method were also lower than LOD and LOQ values reported by Moselhy *et al*.[15] in the determination of MDA in human plasma samples by liquid chromatography coupled with mass spectrometry (0.86 and 2.86 μM).

Accuracy was evaluated from the recovery of MDA standard addition to pooled bovine semen samples. It refers to the difference between the obtained value (mean) and the true value. The basal level of MDA concentration in the sample without standard addition was previously measured and considered. Replicates were processed in different days. Excellent agreement was observed between added and detected MDA concentrations. Mean recoveries were 91 and 90% for HPLC and for spectrophotometric techniques; respectively, as Table 3 shows. Although, these values were statistically different from 100% (P = 0.008 and P = 0.0003), they corresponded to an acceptable recovery values considering the MDA determined in biological samples was in trace levels and being that recovery values as low as 80% are considered normal.[25] Besides, recovery values found in this work were within the range measured by Seljeskog (90-94%) for MDA levels in human serum by HPLC.[26]

Precision, as the degree of concordance between individual assays within homogeneous sample, was determined by triplicate analyses of the samples used in the accuracy assay (Table 3). Repeatability and intermediate precision were evaluated within the same day and in three consecutive days as the interday variability of the proposed method, respectively. The intraday ($P = 0.499$) and interday ($P = 0.729$) relative standard deviations (RSD) were not statistically different between the HPLC and spectrophotometric methodologies and are within acceptable values for biological fluid, since RSD values up to 5 or at most 10% can be accepted.[25]

The long-term stability of MDA-TBA_2 condensation product formed were also evaluated in spiked samples. The degradation of MDA-TBA_2 was estimated by the decrease in MDA concentration determined in cryopreserved bovine semen samples by both HPLC and spectrophotometric techniques after 20 days of storage (Fig. B in Electronic Supplementary Data). The average MDA-TBA_2 degradation decays by HPLC analysis were 17, 9.1 and 12% at 25, 8 and -20 ºC corresponding to room temperature, fridge and freezer storage, respectively. Similar results were observed by spectrophotometric analysis at the same temperatures. In this sense, this assay shows that the TBA-derivatizated samples can be stored until HPLC analysis for eight days with less than 5% decay.

A comparison between MDA measurements by spectrophotometry and HPLC-DAD was undertaken using the validation assays (Table 3), being MDA concentration overestimated by the spectrophotometric technique in respect to HPLC one. This result coincides with that reported by Hong *et al.*[13], who found an approximately 2-fold increase in MDA levels in human plasma samples determined by spectrophotometry compared with those determined by HPLC. This may be

explained considering that other reactive species are able to react with TBA besides MDA. It has been proposed that interferences in MDA spectrophotometric determination may be ascribed to the reaction of TBA with dienals[12] that absorb at the same spectral region of MDA adduct. For this, some weak points such as lack of both sensitivity and specificity have been observed in the spectrophotometric technique due to parallel side reactions. [14]

4. Conclusion

This is the first report of a separative method to determine MDA as an oxidative marker in bovine semen samples. The relevance of determining the most adequate sample pretreatment for this kind of biological material has been demonstrated; our proposal being different from those recommended for other biological fluids whose chemical composition is not the same. Finally, the entire method proposed in this work includes: the procedure A (acidic hydrolysis combined with TBA condensation in a single step) as the best sample pre-treatment followed by reverse-phase HPLC-DAD analysis. The proposed HPLC method is a more selective, sensitive and reproducible analytical technique than the UV-Vis spectrophotometry. This result allows us to evaluate the semen quality previous to insemination artificial process in an early stage. Current HPLC assay resulted in an improvement compared to those previously reported in terms of efficiency of sample preparation because the sample clean-up was not necessary and HPLC column remained without peak broadening more than 50 runs. Moreover, the amounts of sample required could be reduced from mL to μL using HPLC method instead of the traditional spectrophotometric method. This is a great advantage considering that bovine semen samples are extremely valuable and expensive.

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Tables and Figure Captions

Table 1: Efficiency of treatments A (acidic hydrolysis) and B (alkaline hydrolysis) in MDA determination in cryopreserved bovine semen samples.

Table 2: Validation of a new HPLC-DAD method to determine MDA in cryopreserved bovine semen samples comparing with the corresponding traditional spectrophotometric method.

Table 3: Analytical parameter evaluation in the MDA determination in cryopreserved bovine semen samples after acidic hydrolysis (treatment A) by HPLC and spectrophotometry.

Fig. 1: Experimental procedure scheme for MDA analysis in cryopreserved bovine semen by treatments A (total MDA) and B (free and protein bound MDA). Details of the steps depicted here are described in the text.

Fig. 2: Typical HPLC chromatograms of MDA-TBA_2 condensation product. (a) blank; (b) sample; (c) sample + 0.34 μM MDA; (d) sample + 0.68 μM MDA; (e) sample + 1.8 μM MDA.

Fig. 3: Chromatographic elusion of MDA-TBA_2 condensation product at different column temperatures assayed during the optimization of HPLC conditions to determine MDA in cryopreserved bovine samples.

Fig. 4: Typical DAD and UV-Vis calibration curves in the range measured taking into account MDA levels found in cryopreserved bovine semen samples.

Electronic Supplementary Data:

Fig. A: Time profile for hydrolysis of bound protein MDA in treatment B of cryopreserved bovine semen samples. Empty circle (○): HPLC analysis. Full circle (●): Spectrophotometric analysis.

Fig. B: Long-term stability of MDA-TBA_2 condensation product in cryopreserved bovine semen samples using treatment A by HPLC-DAD (1) and the traditional spectrophotometric method (2). Empty circle (○): 25 ºC. Full circle (●): -20 ºC. Half circle ( ): 8 ºC.

Table 1: Efficiency of treatments A (acidic hydrolysis) and B (alkaline hydrolysis) in MDA determination in cryopreserved bovine semen samples.

	Treatment A Total MDA (μM)	Treatment B Total MDA (μM)	Protein bound MDA (μM)	Free MDA (μM)
Spectrophotometry	(0.60 ± 0.02)a	(0.50 ± 0.02)b	0.50 ± 0.02	n.d.
HPLC	(0.54 ± 0.02)a	(0.47 ± 0.02)b	0.38 ± 0.03	0.091 ± 0.009

a and b letters are statistically different values in a row ($P \leq 0.05$), n=6 for each treatment.

n.d.: not detectable (level below LOD and LOQ).

Table 2: Validation of a new HPLC-DAD method to determine MDA in cryopreserved bovine semen samples comparing with the corresponding traditional spectrophotometric method.

Figures of merit	*Analytical Technique*	
	HPLC-DAD	UV-Vis Spectrophotometry
Limit of Detection (LOD, μM)	0.034	0.16
Limit of Quantification (LOQ, μM)	0.086	0.40
Lineal Range (μM)	0.086 - 9.1	0.40 - 5.4
Regression line	$Y = 1.7 \times 10^{5} X - 9.4 \times 10^{3}$	$Y = 1.7 \times 10^{-1} X - 4.7 \times 10^{-2}$
R^2	0.9993	0.9998

Table 3: Analytical parameter evaluation in the MDA determination in cryopreserved bovine semen samples after acidic hydrolysis (treatment A) by HPLC and spectrophotometry.

Methodology	MDA spiked (μM)	MDA found (μM)			Precision (RSD %)				Accuracy (recovery %)		
		Day1 (n=3)	Day2 (n=3)	Day 3 (n=3)	Day1 (n=3)	Day2 (n=3)	Day3 (n=3)	Interday (n=9)	Day1 (n=3)	Day 2 (n=3)	Day 3 (n=3)
HPLC	0	0.34±0.02	0.35±0.02	0.37±0.02	4.9	5.0	4.6	4.8	-	-	-
	0.34	0.66±0.03	0.65±0.03	0.63±0.03	5.0	5.0	5.1	5.0	98	93	89
	0.68	0.95±0.03	0.92±0.04	0.96±0.04	2.7	3.9	3.7	3.4	94	90	92
	1.8	1.88±0.04	1.90±0.05	1.89±0.05	2.0	2.7	2.7	2.5	87	88	87
Spectro-photometry	0	0.40±0.02	0.42±0.02	0.44±0.02	4.7	5.0	4.8	4.8	-	-	-
	0.34	0.70±0.03	0.69±0.03	0.72±0.03	4.9	4.3	4.2	4.5	94	91	93
	0.68	1.00±0.03	0.99±0.03	0.97±0.03	3.1	2.8	2.9	2.9	92	90	87
	1.8	1.99±0.03	1.95±0.02	1.97±0.02	1.3	1.3	1.3	1.3	90	87	88

Values are means ± standard deviation

Fig. 1: Experimental procedure scheme for MDA analysis in cryopreserved bovine semen by treatments A (total MDA) and B (free and protein bound MDA). Details of the steps depicted here are described in the text.

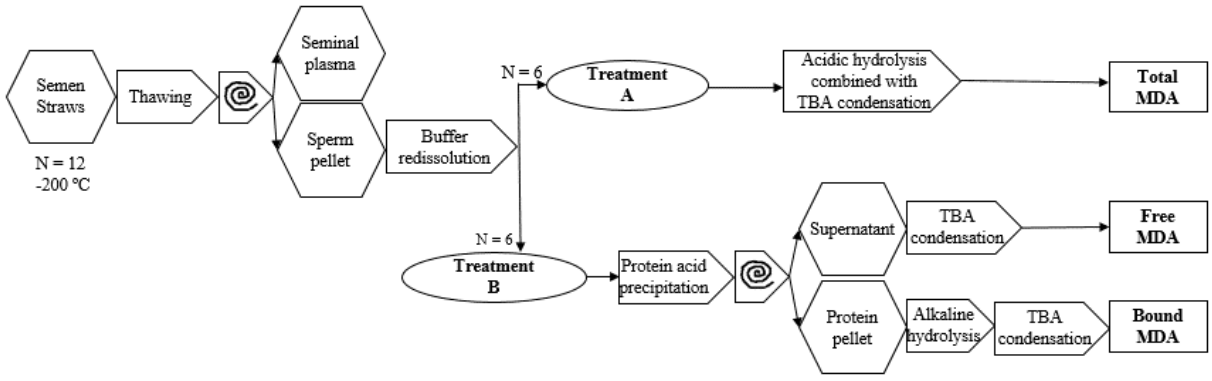

Fig. 2: Typical HPLC chromatograms of MDA-TBA_2 condensation product. (a) blank; (b) sample; (c) sample + 0.34 μM MDA; (d) sample + 0.68 μM MDA; (e) sample + 1.8 μM MDA.

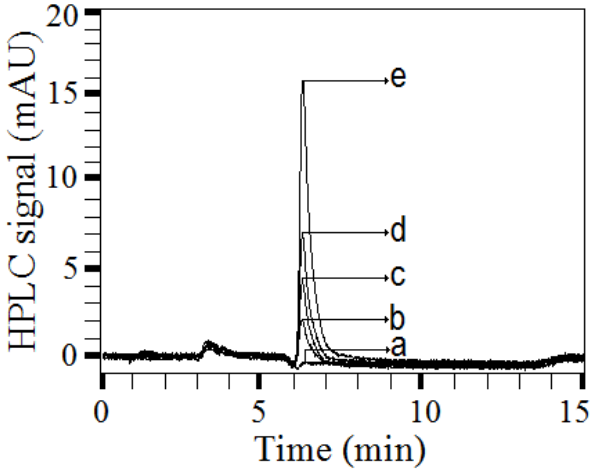

Fig. 3: Chromatographic elusion of MDA-TBA_2 condensation product at different column temperatures assayed during the optimization of HPLC conditions to determine MDA in cryopreserved bovine samples.

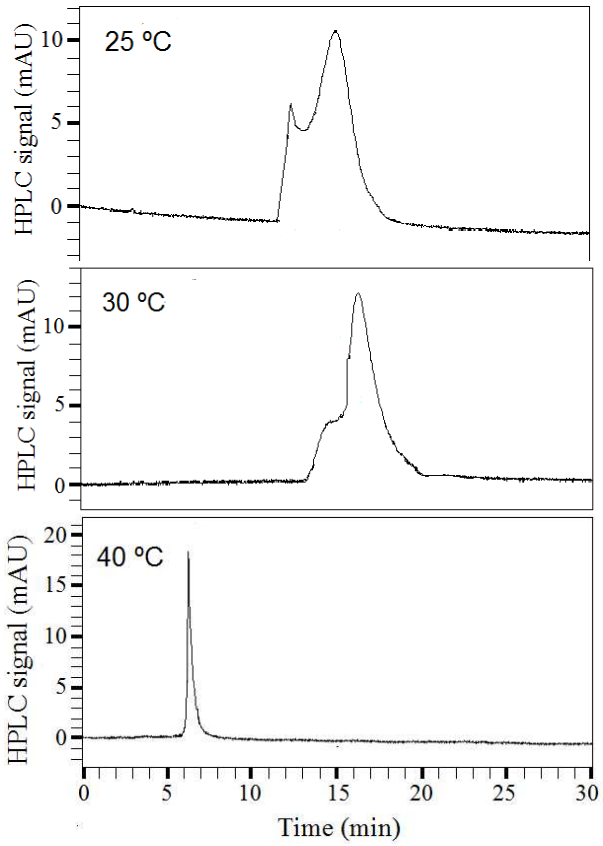

Fig. 4: Typical UV-Vis and DAD calibration curves in the range measured taking into account MDA levels found in cryopreserved bovine semen samples.

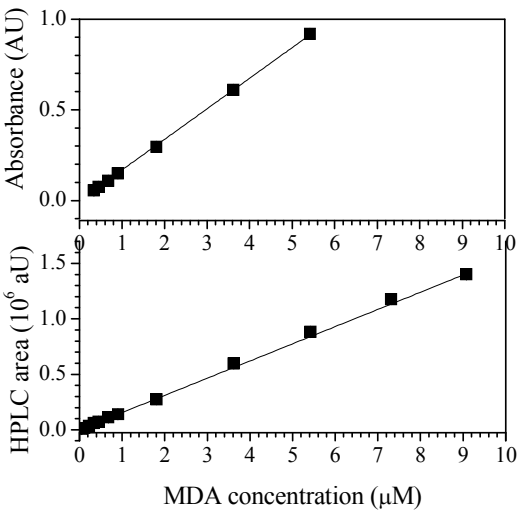

Fig. A Electronic Supplementary Data: Time profile for hydrolysis of bound protein MDA in treatment B of cryopreserved bovine semen samples. Empty circle (○): HPLC analysis. Full circle (●): Spectrophotometric analysis.

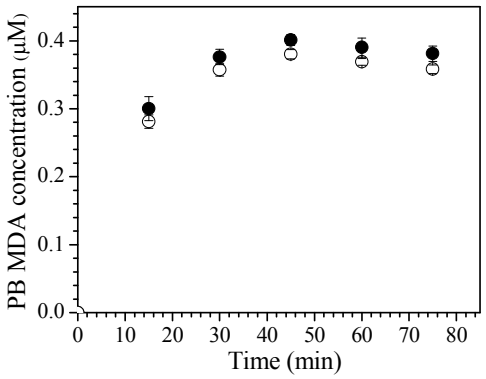

Fig. B Electronic Supplementary Data: Long-term stability of MDA-TBA_2 condensation product in cryopreserved bovine semen samples using treatment A by HPLC-DAD (1) and the traditional spectrophotometric method (2). Empty circle (○): 25 ºC. Full circle (●): -20 ºC. Half circle (◒): 8 ºC.