Tutor/s

Dr. Mercè Granados Juan
Departament de Química Analítica

Asunción Suárez García
Agència de Salut Pública de Barcelona

Grau de Química

Treball Final de Grau

Development and validation of a method for the analysis of erythorbic acid and ascorbic acid in food for human consumption.

Desenvolupament i validació d'un mètode d'anàlisi d'àcid eritòrbic i d'àcid ascòrbic en aliments destinats al consum humà.

Eloi Pulido Bertran

June 2018

UNIVERSITAT DE BARCELONA

B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

L'última cosa que un sap, és per on començar.

Blaise Pascal

Vull agrair a totes les persones que m'han ajudat i m'han donat suport durant aquests mesos, en especial a l'Asunción Suárez i a la Dra. Mercè Granados per la paciència que han tingut, per tot el temps que m'han dedicat, pels consells que m'han donat i per tot el que he après amb elles.

També vull agrair a tots els membres del LASPB per compartir el seu espai i el seu temps amb mi i haver-me permès desenvolupar el treball en el seu centre.

Finalment, agrair a totes aquelles persones del meu entorn, en especial a la meva família, pel seu suport i pels ànims que m'han donat.

REPORT

CONTENTS

1. SUMMARY

Antioxidants are molecules that can prevent other molecules from oxidation. They are used as food additives in all kind of foodstuffs to lengthen their period of consumption. For different meat foodstuffs, ascorbic acid (AA) and erythorbic acid (EA) are the most used antioxidants.

AA is not restricted as food additive and it can be added as much as its necessary, but the use of EA as food additive in meat matrixes cannot be higher than 500 mg/Kg (MRL). Liquid chromatography (LC) is the chosen technique for the analysis of these compounds in complex matrixes. The most conventional modalities of reversed phase do not give good resolution between AA and EA.

The main objective of this study is to establish a method based on liquid chromatography coupled with absorption spectrophotometry for the separation and quantification of AA and EA at concentrations near or below 500 mg/Kg.

The developed method is based on hydrophilic interaction liquid chromatography (HILIC), a modality of chromatography especially suitable for very polar compounds, which use is growing in the last years but is not as stablished as reversed phase modality. Otherwise, the analytes tend to oxidise in aqueous solution.

Previous studies have been carried out: stability tests to avoid degradation of AA and EA and chromatographic conditions tests to achieve the optimal conditions for the separation and quantification of both analytes.

Extraction recovery, linearity, precision, trueness and uncertainty of the established method have been determined, and the results of the validation are fully satisfactory. The method allows the confirmatory analysis of the analytes from 30 mg/Kg (LOQ) to concentrations higher than 500 mg/Kg (MRL).

Keywords: Erythorbic acid, ascorbic acid, HILIC, UV detection, food analysis, processed meat samples, antioxidants.

2. RESUM

Els antioxidants són molècules que prevenen l'oxidació d'altres molècules. S'utilitzen com a additius alimentaris en tot tipus d'aliments per tal d'allargar el seu període de consum. L'àcid ascòrbic (AA) i l'àcid eritòrbic (EA) són els antioxidants més utilitzats en diferents tipus de carns.

L' AA no té restringit el seu ús com a additiu alimentari i es pot addicionar en l'abundància que es cregui convenient, però l'ús de l'EA no pot sobrepassar els 500 mg/Kg (MRL) en matrius càrniques. La cromatografia de líquids (LC) és la tècnica escollida per a l'anàlisi d'aquest compostos per matrius complexes. Les modalitats més convencionals de fase inversa no proporciona una bona resolució entre l'AA i l'EA.

L'objectiu principal d'aquest treball ha estat establir un mètode basat en la cromatografia de líquids acoblada amb espectrofotometria d'absorció per la separació i la quantificació d'AA i EA en concentracions properes i inferiors a 500 mg/Kg.

El mètode que s'ha desenvolupat es basa en la cromatografia de líquids d'interacció hidrofílica (HILIC), una modalitat especialment adequada per a compostos molt polars i que s'està estenent aquests darrers anys, però que no està tant establerta com la modalitat de inversa. D'altra banda, els analits tenen tendència a l'oxidació en dissolució aquosa.

S'han fet estudis d'estabilitat per tal de conéixer la degradació de l'AA i de l'EA, i s'ha fet un estudi de les condicions cromatogràfiques òptimes per la separació i quantificació dels dos anàlits.

S'ha validat el mètode avaluant el rendiment de l'extracció, la linealitat, la precisió, la veracitat i la incertesa del mètode que s'ha establert i els resultats de la validació són plenament satisfactoris. El mètode permet l'anàlisi confirmatòria dels anàlits des de 30 mg/Kg (LOQ) fins concentracions superiors a 500 mg/Kg (MRL).

Paraules clau: Àcid eritòrbic, àcid ascòrbic, HILIC, detecció UV, anàlisi d'aliments, mostres de carn processada, antioxidants.

3. INTRODUCTION

In biological systems, antioxidants protect against oxidative damage and in human beings can help to prevent cardiovascular, neurological and/or carcinogenic diseases [1]. Ascorbic acid (AA), also known as vitamin C, is a natural antioxidant in biological systems, and it is involved in several biochemical mechanisms, such as collagen synthesis, immune response, pulmonary function and iron absorption [2,3,4].

AA is naturally present in most food commodities. In contrast, erythorbic acid (EA), also known as isoascorbic acid, does not exist naturally in food [2]. EA is the D-isomer and C-5 epimer of AA [4,5,6]. Because of their structural similarities, they are expected to have very similar physic-chemical properties. Both are very polar small molecules and they easily oxidize in aqueous solution. Parameters that can intensify degradation are light, increased temperature, increased pH, low concentration and presence of oxygen or metal ions.

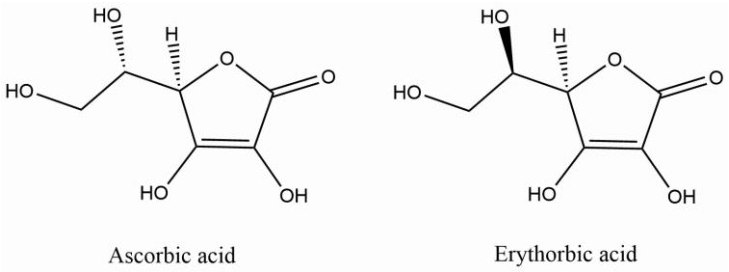

Figure 1: Ascorbic acid and erythorbic acid molecules structure.

AA and EA rapidly oxidize to dehydroascorbic acid (DHAA) and dehydroerythorbic acid (DHEA), respectively. These reactions are reversible, and they are the key to the antioxidant activity of AA and EA.

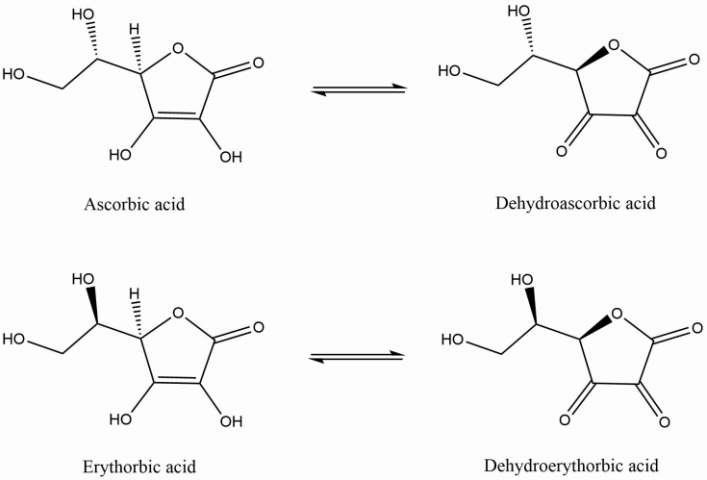

Figure 2: Ascorbic acid and erythorbic acid oxidation to dehydroascorbic acid and dehydroerythorbic acid.

Further oxidation generates 2,3-diketo-L-gulonic acid (DKG) and 2,3-diketogluconic acid (DKGluA), respectively, which have no biological function and the reactions are no longer reversible [2].

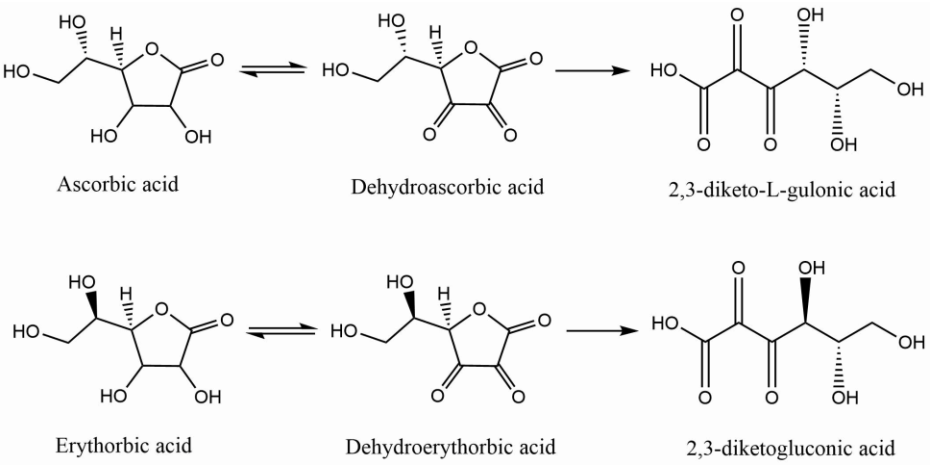

Figure 3: Oxidation reactions of ascorbic acid and erythorbic acid.

Ascorbic acid (E 300), sodium ascorbate (E 301), calcium ascorbate (E 302); as well as erythorbic acid, also known as isoascorbic acid (E315), sodium erythorbate (E 316); are antioxidants used as food additives in order to prevent degradation of food during the packaging time [1]. The addition of antioxidants is allowed in foodstuffs. However, their use is restricted by European Union (EU) regulations (Regulation EC 1333/2008). On the one hand, the use of AA as food additive is permitted at *quantum satis* (as much as is sufficient) in most food commodities, although it is regulated for some baby foods at 200 and 300 mg/Kg [7]. The recommended daily allowance for AA is 60 mg/day (2009) [6]. On the other hand, the use of EA as food additive is allowed for only a few foodstuffs; it is restricted to 500 mg/Kg (MRL) in some processed meat matrixes and 1500 mg/Kg in some fish matrixes. The acceptable daily intake (ADI) is 6 mg/kg bw/day [8]. Appendix 1 and Appendix 2 show the maximum levels permitted of use of AA and EA as a food additive in EU [7,8].

Unexpected occurrence of EA has been found in products such as beer, wine, baby foods, etc., where EA is used rather than AA as an antioxidant, probably because EA is cheaper than AA [2].

3.1. METHODS OF ANALYSIS

Analysis of AA and EA in food basically requires the extraction of the analytes from the matrix and the quantitative determination by liquid chromatography.

3.1.1. Extraction

No complex extraction techniques are required to extract AA and EA from the samples. In general, these analytes can be extracted by simple shaking, after adding a volume of an extraction solution. According to the literature, the extraction solution is usually oxalic acid or m-phosphoric acid (MPA) aqueous solution. In some cases, dithiotreitol (DTT), EDTA or tris-2-carboxyethylphosphine (TCEP) aqueous solutions have also been used. These compounds can stabilize AA and EA from degradation before the chromatographic separation.

Most authors do not apply any clean-up to remove interferences before chromatographic separation.

Table 1 summarizes extraction methods applied for the analysis of AA and EA in food samples.

Table 1: Survey of extraction methods for AA and EA analysis in different food samples.

Analyte	Sample	Extraction	Clean-up	Ref.
AA and EA.	Fish tissue.	Oxalic acid 10 mM solution. Sonication.		[2]
AA and DHAA.	Vegetables and fruits.	0.05% (w/v) EDTA solution. Mechanical shaking.	C18 cartridge.	[3]
AA, EA, AA-2G and AA-2BG.	Tea drinks, dried fruit.	Acetonitrile-66.7 mM ammonium acetate aqueous solution containing 50 mg/l DTT aqueous solution (85:15, v/v). Sonication.		[4]
AA, EA, DHAA and DHEA.	Foodstuffs.	Aqueous solution 1% (w/v) MPA and 0.5% (w/v) oxalic acid. If high content of starch, 2% (w/v) MPA and 1% (w/v) oxalic acid. Mechanical shaking.		[5]
AA, EA, DHAA and DHEA.	Fruit juices freeze dried and ground chestnuts, ham.	Solution of 5% MPA, 2 mM TCEP and 2 mM EDTA. Mechanical shaking.		[6]
AA and EA	Fruit juice.	10 % MPA solution. Mechanical shaking.		[9]
AA	Fruits and vegetables.	Extraction with 3% MPA aqueous solution and addition of a solution of EA (internal standard) dissolved in 0.2 % DTT aqueous solution. Mechanical shaking.		[10]

The method proposed by Barros et al. [6] is the only one found in the literature that is applied to determine ascorbic acid and erythorbic acid in a meat matrix (ham).

3.1.2. Chromatographic analysis

Reversed phase liquid chromatography (RP-LC) is not adequate to separate AA and EA because they are very polar compounds and they are very weakly retained. An alternative to analyse AA and EA is hydrophilic interaction liquid chromatography (HILIC), a modality to analyse polar compounds that are unretained or weakly retained in reverse phase liquid chromatography (RP-LC) [2,4,11,12]. In 1990, Alpert used this term to describe the use of polar stationary phases with aqueous-organic mobile phases, with a high content of the organic solvent [2,13].

3.1.2.1. HILIC mechanism

HILIC retention mechanism is partitioning. There are two phases, an organic-solvent-rich mobile phase and a water-rich layer absorbed onto the stationary phase surface. Equilibrium of the analytes between these two layers occurs. The more hydrophilic (or polar) the analyte, the more concentration of this analyte in the water-rich later absorbed onto the stationary phase and the more retention of the analyte, but often there are other mechanisms involved [14]. Electrostatic interactions can be significant whether the stationary phase and the analyte are both charged. Hydrogen bonding can also contribute to the mechanism. Interaction of basic and acidic analytes with the stationary phase is expected to be based on a superposition of both hydrophilic interactions and electrostatic forces. In conclusion, HILIC retention mechanism is more complicated than partitioning or ion-exchange chromatographic mechanisms. Depending on the type of stationary phase or mobile phase (pH, ionic force, type and concentration of salt, and organic solvent content), a prevalence of a mechanism among others occurs [2].

3.1.2.2. Stationary phase

Different types of polar stationary phases are used in HILIC. Diol and aminopropyl bonded to silica columns are some of the most used. On the one hand, diol columns mostly base the retention mechanism in hydrogen bonding properties and can show good retention for polar compounds. On the contrary, despite amino propyl columns can retain all kind of polar molecules, acidic molecules are more strongly retained because of electrostatic interactions when the working pH is higher than the pKa of the analyte [14].

3.1.2.3. Mobile phase

Mobile phases used in HILIC consist on aqueous-organic mixtures of solvents where, typically, acetonitrile is the organic solvent (mobile phase content higher than the 85%) and water is the strong solvent. Gradient and isocratic conditions can both be performed in HILIC, although isocratic conditions are more common in the literature.

Usually, the mobile phase can carry ionic additives, which are used to control pH and ionic strength. These parameters can contribute in different degree to the retention of the analytes.

Control of pH can be important to ensure a specific ionic form of the analyte during the analysis. However, the more concentration of the ionic additive, the less retention of the analyte if ion exchange controls the retention [14].

3.1.2.4. Chromatographic separation of AA and EA

Currently, HILIC coupled to ultraviolet detection (UV) is the most applied chromatographic mode for the separation and quantification of AA and EA. Despite the stationary phase of the columns found in the literature is varied, diol and aminopropyl bonded to silica and amide gel columns are the most used. High content of acetonitrile, due HILIC mode, is proposed in most references and the most common mobile phase ionic additive is ammonium acetate.

Table 2 summarizes chromatographic methods applied for the analysis of AA and EA in food samples.

Table 2: Survey of chromatographic conditions for the separation of EA and AA.

Analytes	Column	Mobile phase	Detection	Ref.
AA and EA	APS-2 Hypersil.	Acetonitrile-ammonium acetate 100 mM (90:10, v/v)	UV (240 nm) and MS/MS	[2]
AA, EA, AA-2G and AA-2BG	Inertsil Diol	Acetonitrile-water-formic acid (90:9.5:0.5, v/v)	UV (240 nm)	[4]
AA, EA, DHEA and DHAA	Jupiter C18, Phenomenex	2.3 mM dodecyltrimethylammonium chloride and 2.5 mM Na_2EDTA in a 66 mM phosphate-20 mM acetate buffer (pH=4.50)	UV (247 nm), electrochemical and Fluorescence (after post column derivatization)	[5]

AA and EA, total content (DHAA and DHEA)	TSK Amide-80	Acetonitrile-0.1% TFA (90:10, v/v; changed to 80% of 0.1% TFA solution after 7 min of analysis and changed to 10% of 0.1% TFA solution at minute 14 until analysis).	UV (244 nm)	[6]

Note that AA and EA have been analysed in many food commodities. Nonetheless, ham and fish tissue are the only meat-type matrixes that have been analysed. Analysis methods for processed meat products were not found.

4. OBJECTIVES

The objective is to develop an LC method (HILIC mode) with UV detection for the separation and quantification of ascorbic acid and erythorbic acid in processed meat products such as cured-sausages (sausages and chorizo).

The specific objectives of this study are:

- To develop a LC method (HILIC mode) with UV detection for the analysis of AA and EA.
- To set up a method for the extraction of AA and EA from processed meat foods.
- Validate the analytical methodology for the analysis of AA and EA in processed meat samples.

5. EXPERIMENTAL SECTION

5.1. MATERIALS AND INSTRUMENTATION

5.1.1. Standards

AA was supplied as L-(+)-ascorbic acid by Panreac (Barcelona, Spain) and EA as sodium D-isoascorbate monohydrate 97% was supplied by Sigma-Aldrich (Steinheim, Germany). A mixture of EA and AA in 10 mM oxalic acid aqueous solution at a concentration of 1000 mg/l is used to prepare calibration standards and to spike samples in recovery experiments. This solution is stored at 4 ºC and it is stable for 4 days.

5.1.2. Reagents and solvents

The reagents and solvents used are: oxalic acid (Sigma Aldrich, Steinheim, Germany), m-phosphoric acid (ACRŌS, Germany), tert-butylhydroquinone (t-BHQ) (Fluka), propyl gallate (Fluka, Steinheim, Germany), ammonium acetate (MERCK, Darmstadt, Germany), acetonitrile (Honeywell, Seelze, Germany) and Milli-Q water (Millipore, England).

5.1.3. Instruments and laboratory material

An UHPLC (Agilent Technologies 1290 Infinity II) with automatic injector (G7167B 1290 Multisampler), binary pump (G4220A 1290 Bin Pump), column oven (G1316C 1290 TCC); coupled with a diode array detector (G4212A 1290 DAD) (UHPLC-DAD).

Other used instruments were: Analytical balance (Sartorius SECURA224-1S), precision balance (METLER TOLEDO XPE3003S), a centrifuge (Sorvall Lynx 6000 Centrifuge Thermoscientific), a multivortex mixer (VWR DVX-2500 Multi-Tube Vortexer), a vortex mixer (IKA VORTEX GENIUS 3) and an ultrasound bath (P-SELECTA ULTRASONS-H).

Laboratory material has also been used: beakers of 100 mL to 250 mL, vials, amber tubes to centrifuge, amber glass tubes, automatic pipettes, Pasteur pipettes, volumetric flasks of 10 mL, membrane filters (0.22 µm, Durapore, PVDF) and other materials as spatula, gloves, etc.

5.2. DETERMINATION OF AA AND EA

5.2.1. Extraction process

2 g of sample weighed in a precision balance are mixed with 10 mL of oxalic 10 mM aqueous solution into a centrifuge tube. The mixture obtained is mixed using a multivortex during 1 minute at 2500 rpm. Then, the tubes are sonicated into an ultrasound bath at a temperature between 4 and 10 ºC for 5 minutes. The sonicated extracts are centrifuged at 12000 rpm, at 4ºC during 15 min. After that, 1 mL of the extract is mixed with 1 mL of a solution of 200 mg/L t-BHQ in acetonitrile and mixed on a Vortex. Finally, the mixture is filtered through a 0.22 µm membrane filter.

A minimum of one spiked sample must be carried out to each series of analysis as quality control.

It is important to preserve temperature at 4 ºC during extraction process to avoid degradation of the analytes.

5.2.2. HILIC-UV analysis

AA and EA analysis were performed using UHPLC chromatograph (Agilent Technologies 1290 Infinity II) coupled to diode array detector.

An aminopropyl bonded to silica column, APS-2 Hypersil (50 mm x 2.1 mm i.d., 3 µm), was used for the chromatographic separation. Isocratic eluting conditions were performed using acetonitrile-ammonium acetate 50 mM at pH 6.5 (90:10) as the mobile phase, with a flow rate of 0.4 $mL \cdot min^{-1}$. Temperatures of the injector and the column were set to 4ºC and 20 ºC, respectively. Detection of the analytes was based on UV absorption, using 270 nm as the absorption wavelength for both analytes (maximum absorption). A chromatographic analysis takes 10 min and retention times for EA and AA are, approximately, 4.6 min and 6.9 min, respectively.

Examples of chromatograms of a solution of the standards and a chorizo sample spiked at 800 mg/Kg are shown in Figure4 and Figure 5, respectively.

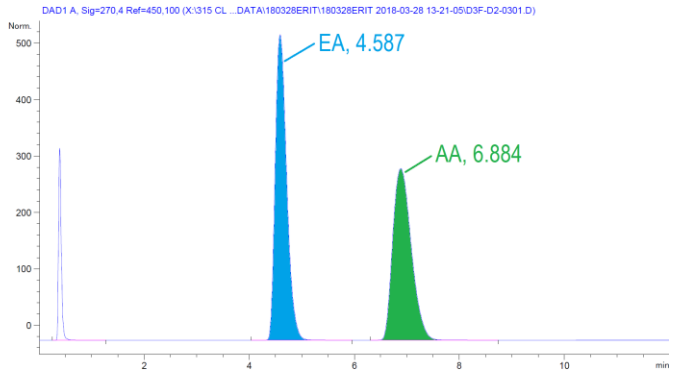

Figure 4: AA and EA HILIC-DAD chromatogram at 90:10 ACN-50 mM ammonium acetate pH 6.5, aminopropyl stationary phase. Standard chromatogram.

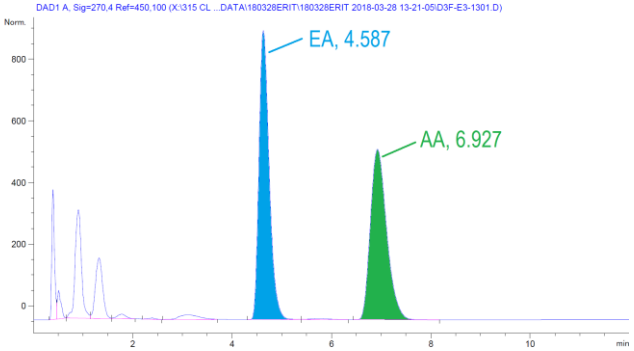

Figure 5: AA and EA HILIC-DAD chromatogram at 90:10 ACN-50 mM ammonium acetate pH 6.5, aminopropyl stationary phase. Sample chromatogram.

5.2.3. Calibration

Calibration standards were prepared by dilution of the 1000 mg/L stock solution of AA and EA. A 100 mg/l standard solution of AA and EA was done in order to prepare the smallest concentrations of the calibration curves. Due to problems with linearity, three calibration curves in different ranges are performed:

- From 6 to 20 mg/L, low level. This curve is suitable for the analysis of concentrations from 30 mg/L to 100 mg/L in sample. Prepared from the 100 mg/L solution.
- From 30 to 80 mg/L, medium level. This curve is suitable for the analysis of concentrations from 100 mg/L to 450 mg/L in sample. Prepared from the 1000 mg/L solution.
- From 90 to 300 mg/L, high level. This curve is suitable for the analysis of concentrations from 450 mg/L to 1500 mg/L in sample. Prepared from the 1000 mg/L solution.

All standards were prepared using amber volumetric flasks.

The standards are prepared daily, and they must be quickly placed at 4 ºC until the sequence starts.

6. RESULTS AND DISCUSSION

6.1. STUDY OF STABILITY OF STANDARDS

A study of degradation of the 1000 mg/L stock solution was performed to assess the period that can be used for this function. A 1% MPA aqueous solution, 5% MPA aqueous solution and 10 mM oxalic acid aqueous solution have been tested as stabilizers of AA and EA solutions in periods of 1 day. The stock solutions are preserved inside the fridge. The degradation is considered relevant after the peak area has decreased more than 15% of the first day peak area.

The chosen stabilizer solution for conservation AA and EA of the stock solution is also used to extract the analytes from the samples.

Table 3 shows the peak area results of AA and EA each day of analysis for each stabilizer solution. Problems with the pressure stability during chromatography caused a loss of data at the 3rd and 4th days of the study for 1% MPA and 5% MPA solution, and at the 9th day for 10 mM oxalic acid.

Table 3: Stability study of different stabilizers for the preservation of AA and EA.

Stabilizer	Analytes	Peak area (oxalic acid)				
		Day 1	Day 2	Day 3	Day 4	Day 9
1% MPA	AA	8176.90	7567.06			6352.18
	EA	8598.25	7907.93			5228.16
5% MPA	AA	8412.30	8036.31			6643.52
	EA	8770.64	8342.64			5590.12
10 mM oxalic acid	AA	7673.51	7921.85	7403.95	6814.45	
	EA	7968.24	8142.82	7677.73	7388.10	

It has been observed that oxalic acid solution preserves the analytes from oxidation better than MPA solutions because oxalic acid solution shows a loss of peak area smaller than the MPA solutions through during the firsts days of the test. Note that 10 mM oxalic acid solution 2nd day peak area is very similar to the 1st day peak area, and for 1% and 5% MPA solutions a loss of, approximately, 10 and 5% of peak area is observed, respectively, at the 2nd day of study. At the 4th day, a loss of 11% and 7% of peak area is observed for AA and EA preserved in 10 mM oxalic acid solution, respectively. This period is the maximum preservation time for these analytes.

At the 9th day, a loss of 22 % and 39% of peak area has been noticed for AA and EA in 1% MPA solution, respectively, and a loss of 21% and 36% of peak area for AA and EA in 5% MPA solution, respectively. After this time, they not fulfil the condition stablished and they are no longer useful for the calibration preparations.

It has been detected that peak area given by the standards preserved with 1% MPA and 5% MPA are a little higher than the obtained with oxalic acid. Otherwise, the loss of area through days is bigger for the standards preserved with the MPA solutions. So, they cannot stabilize AA and EA at the same degree as oxalic acid. MPA solutions also cause fluctuations in the pressure and, in consequence, in the retention times during the chromatography.

The 10 mM oxalic acid aqueous solution is chosen for the preservation of the stock solution and for the extraction of AA and EA because it can preserve better than MPA solutions and provide constant conditions during chromatography. It can be used for the 4 days from the day of its preparation.

6.2. OPTIMIZATION OF THE CHROMATOGRAPHIC SEPARATION OF AA AND EA

Because of the complexity of HILIC mechanisms, little changes in the chromatographic conditions can lead to significant changes in the retention mechanism. For this reason, a study of chromatographic conditions was carried out.

Two columns were tested in this work: diol KINETEX HILIC (150mm x 3 mm i.d., 2.6 µ) and aminopropyl APS-2 Hypersil (50mm x 2.1 mm i.d., 3µ). For both columns, pH, concentration of ionic buffer, and organic solvent percentage in the mobile phase were tested.

6.2.1. Mobile phase organic solvent content

Figure 6 shows the influence of the organic content of the mobile phase in HILIC chromatography fo a diol stationary phase.

A slight increase of aqueous content in the mobile phase leads to a relevant decrease of retention and resolution of the analytes. For both columns, it can be noticed that modifications of 2% of the organic content of the mobile phase lead to completely different retention times and can increase resolution considerably.

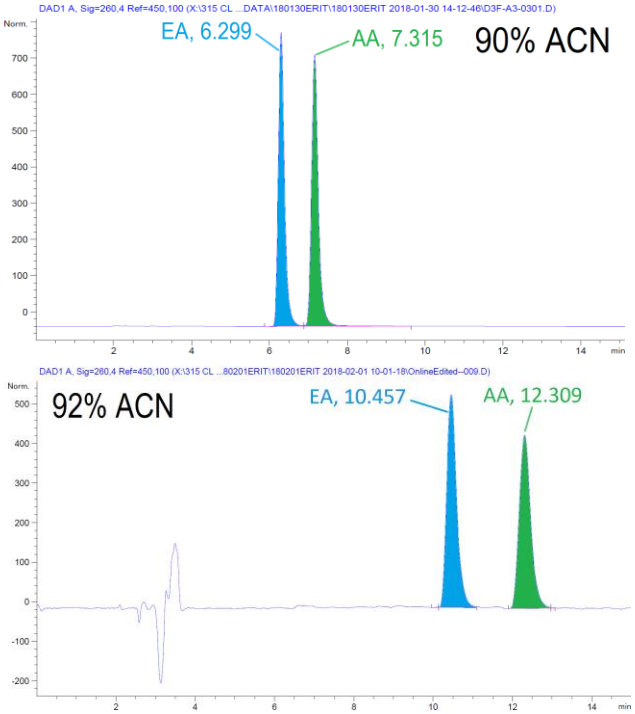

Figure 6: AA and EA HILIC-DAD chromatograms at pH 3.6, 100 mM ammonium acetate aqueous solution. Stationary phase: diol. Mobile phase composition: 90% ACN and 92% ACN.

Figure 7 shows the influence of the organic content of the mobile phase in HILIC chromatography for the aminopropyl stationary phase. Note that flow rate in 87% ACN chromatogram is lower than in 85% ACN chromatogram. Although this difference in the flow rate, 85% ACN in the mobile phase elute the analytes before 87% ACN, compensating the differences of flow rates. This fact shows the strong eluting power of water content in the mobile phase.

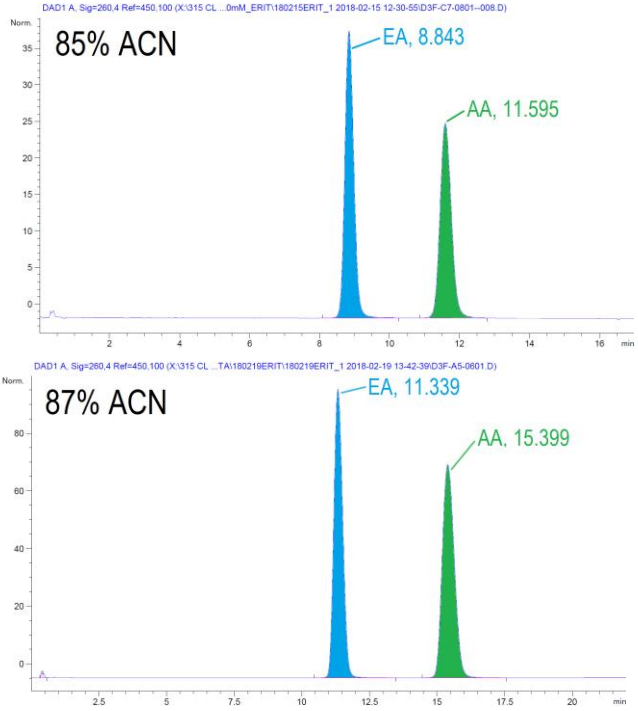

Figure 7: AA and EA HILIC-DAD chromatograms at pH 6.5, 100 mM ammonium acetate aqueous solution, Sationary phase: aminopropyl. Mobile phase composition: 85% ACN and 87% ACN. Flow rate of 0.3 mL/min and 0.4 mL/min, respectively.

6.2.2. Mobile phase pH

Figure 8 and Figure 9 show the influence of pH in diol stationary phase and aminopropyl stationary phase, respectively. Ammonium acetate aqueous solution pH is adjusted with acetic acid to get pH 3, 3.6 and 4. pH 6.5 is achieved with the ammonium acetate solution without adjustment.

An increase of retention of the analytes is also observed with the increase of pH, especially in the aminopropyl column. As in diol column there are not any ionizable groups, the mechanism is partitioning. In the aminopropyl column, it is much more significant because there is a change in retention mechanism from partitioning (low pH) to ion-exchange (high pH) when pH increases at values similar or higher than 4.17 and 4.04 (pKa of AA and EA, respectively [7,8]), conditions at the analytes are both negatively charged and they can strongly interact with positively charged stationary phase.

Also, the more pH, the more resolution is observed in both columns. At the same conditions, aminopropyl stationary phase show higher resolution than diol stationary phase. The resolution for aminopropyl stationary phase at pH 4 is 4.2 and, for diol stationary phase at the same pH, is 2.8. At pH 3 is also observed the same (1.1 for aminopropyl column and 0.60 for diol column).

In diol column, at pH values lower than 4, resolution between AA and EA is not satisfactory as its values are below 1.5 (0.6 and 1.3 for pH 3 and 3.6, respectively).

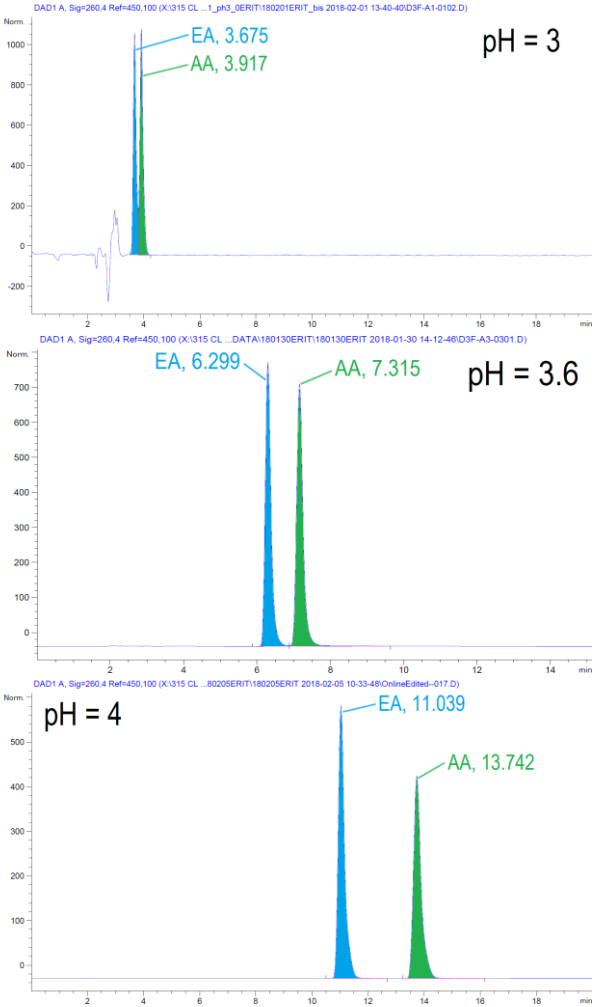

Figure 8: AA and EA HILIC-DAD chromatograms at 90:10 acetonitrile-100 mM ammonium acetate aqueous solution. Diol column: pH 3, 3.6 and 4.

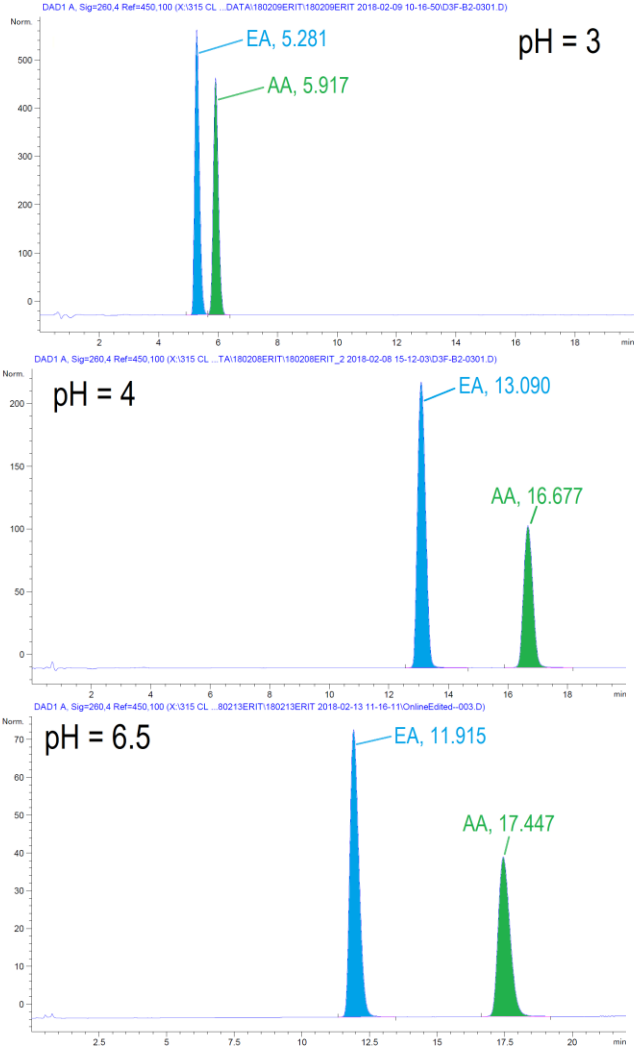

Figure 9: AA and EA HILIC-DAD chromatograms at 90:10 acetonitrile-100 mM ammonium acetate aqueous solution. Aminopropyl column: pH 3, 4 and 6.5.

6.2.3. Mobile phase ionic strength

Figure 10 shows the influence of ionic strength in the retention of AA and EA. This parameter was only tested for aminopropyl column because in the literature was found that that ionic strength in non-charged columns, such as diol columns, has no relevant influence in retention mechanism.

An increase in the ionic strength of the mobile phase at pH 6.5 leads to a decrease of retention, due a prevalence of electrostatic interactions in retention mechanism among others. This behaviour agrees with what has been reported by Drivelos et al. [2]. Low ionic strength can lead to fluctuations of the retention time.

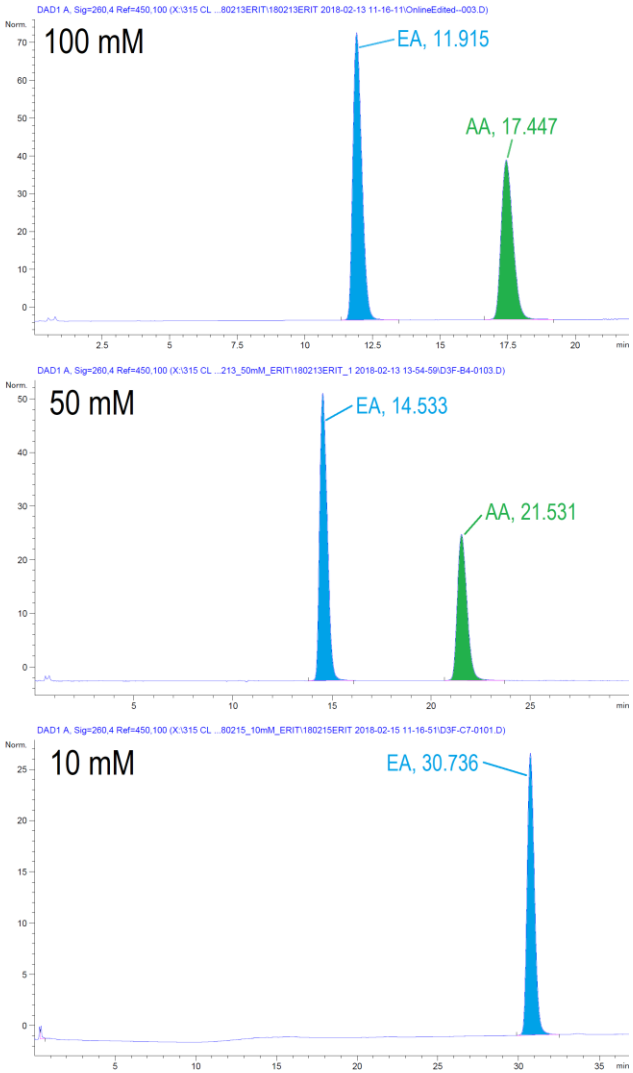

Figure 10: AA and EA HILIC-DAD chromatograms at 90:10 acetonitrile-ammonium acetate aqueous solution pH = 6.5. Aminopropyl column. Ionic strength: 100 mM, 50 mM and 10 mM.

Chromatograms longer than 35 min are obtained at 10 mM of ionic strength. This chromatography time is considered too long for the analysis of AA and EA.

6.2.4. Column temperature

Figure 11 shows the influence of temperature of the column oven for the analysis of AA and EA. Very similar retention times were observed at 15 ºC and at 20 ºC. For this reason, temperature is considered not to have relevant influence in retention mechanism. Although no relevant differences in retention times are observed, no constant pressure during chromatography at 15 ºC is achieved, maintaining retention times less constant when a long chromatographic sequence of injections was carried out.

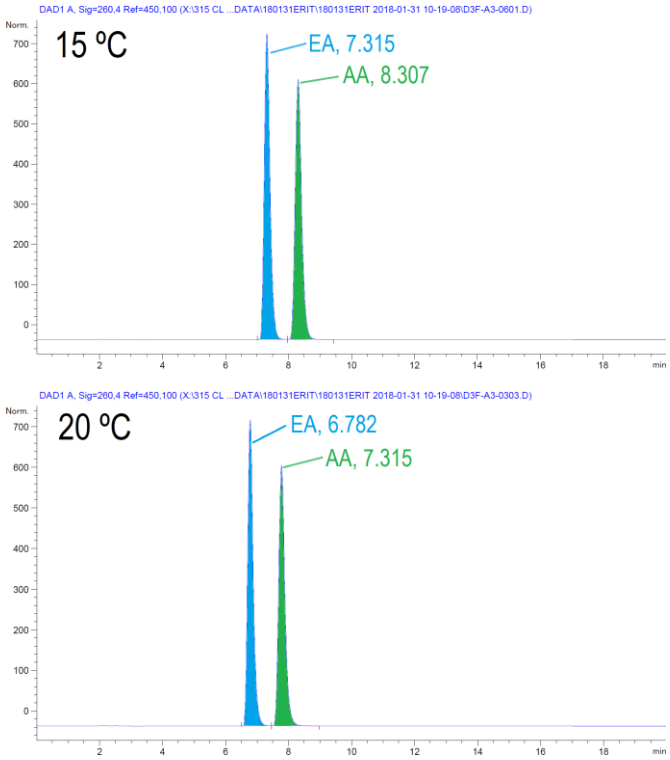

Figure 11: AA and EA HILIC-DAD chromatograms at 90:10 acetonitrile-100 mM ammonium acetate pH 3.6. Diol column. Column temperature: 15 ºC and 20 ºC.

6.2.5. Optimal conditions

Optimal conditions for diol column have been achieved using as mobile phase a mixture of acetonitrile-100 mM ammonium acetate aqueous solution pH 4 (90:10, v/v), flow rate 0.3 mL·min^{-1}, injection volume of 2 µL, with a resolution of 2.8 and leading to a chromatogram of 15 min. A chromatogram at these conditions is shown in Figure 12.

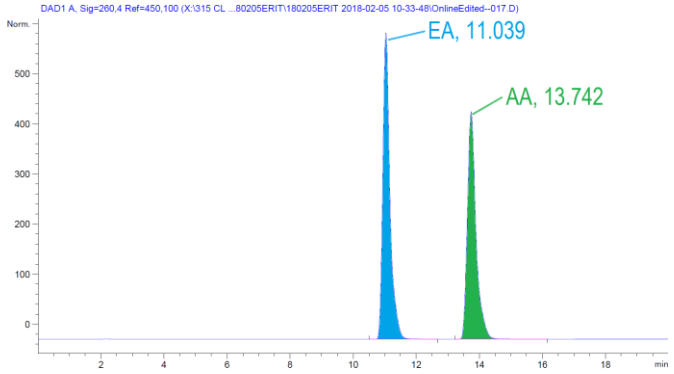

Figure 12: Optimal conditions for the determination of AA and EA using a diol stationary phase.

Optimal conditions for aminopropyl column have been achieved using as mobile phase a mixture of acetonitrile-50 mM ammonium acetate pH = 6.5 (90:10, v/v), flow rate 0.4 mL·min^{-1}, injection volume of 3 µL, with a resolution of 2.9 and leading to a chromatogram of 10 min. A chromatogram at these conditions is shown in Figure 13.

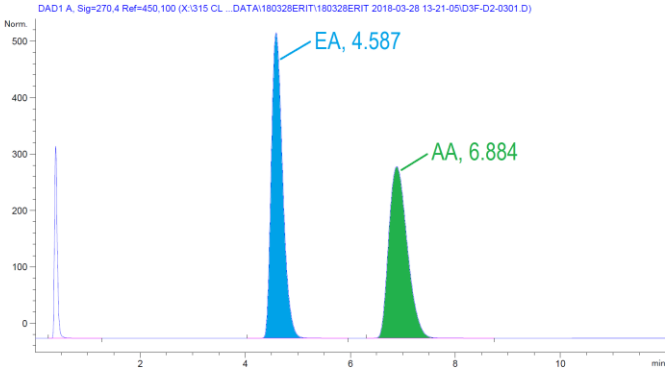

Figure 13: Optimal conditions for the determination of AA and EA using an aminopropyl stationary phase.

Both columns could have been acceptable for the analysis of AA and EA. APS-2 Hypersil (amonipropyl) column was chosen because retention times remain more constant than obtained in diol column, and higher resolution is achieved in less time of analysis

6.3. OPTIMIZATION OF EXTRACTION

Extraction of AA and EA, due their very high polarity, must be extracted with an aqueous solution containing a stabilizing agent. As described in sections 5.2.1. and 6.1 and according to the method described by Drivelos et al. [2], the extraction was performed with a 10 mM oxalic acid aqueous solution.

Some extraction parameters have been tested to optimize the extraction procedure and the chromatographic analysis; such as extractant volume, temperature of extraction and a dilution of the extract. The addition of an antioxidant to the dilution volume was also assessed.

6.3.1. Extraction solvent volume

At the firsts test, 20 mL of oxalic acid aqueous solution was used to extract analytes from the samples. In order to improve the LOQ, it was proposed to test a volume of 10 mL of the 10 mM oxalic acid aqueous solution instead of 20 mL. No relevant differences in the order the magnitude of peak areas were observed performing the extraction with 20 and 10 mL of the oxalic acid solution. Also, it was observed that extraction with 10 mL compensate the area decreased with the dilution of the extractant using an acetonitrile solution, specified in the section 5.2.1.

10 mL was chosen as a final volume for the extraction.

6.3.2. Extraction temperature

The working temperature during the extraction process may be a critical parameter that can influence in the recoveries obtained with the extraction process. Extraction temperature was tested by comparing the recovery values of the analytes obtained by maintaining ultrasound bath, centrifuge and extraction solutions at room temperature and maintaining this equipment at 4 ºC. Three spiking levels were tested: at 30 mg/L (LQ), 100 mg/L and 800 mg/L.

Average recovery percentages of extraction at 4ºC and at room temperature are shown in Table 4. Recoveries of, approximately, 90% are observed at room temperature. These recoveries are a 5-9% lower than at 4 ºC.

Table 4: Recoveries for EA and AA at different extraction temperatures.

Analyte	Recovery at room temperature (%)	Recovery 4 ºC (%)
AA	90.2	95.4
EA	87.3	95.8

Not controlling the temperature during a step of the extraction procedure is not a critical parameter for the recovery, as the recoveries obtained at room temperature are acceptable by the LASPB protocol. However, it is recommended to perform the extraction step at 4 ºC because there are more critical parameters that can decrease the recovery of the extraction process.

The chosen temperature of extraction process is 4 ºC to ensure optimal extraction.

6.3.3. Dilution of the extract and addition of an antioxidant

During the first tests with standards at optimal conditions, relevant instability of pressure was noted during a chromatographic sequence of injections, which leaded to a movement of the retention times and the sudden stop of the sequence of injections due to overpressure. It was perceived that a high difference in composition between the injected sample and the mobile phase may be a determinant parameter for the instable pressure. For this reason, a dilution before injection of aqueous extracted solution with organic solvent (acetonitrile or a mixture of acetonitrile and ammonium acetate aqueous solution (1:1, v/v)) was tested in order to maintain the pressure constant.

The mixture of acetonitrile and ammonium acetate aqueous solution reduced instability of the pressure, but pressure did not remain constant. Otherwise, dilution with acetonitrile maintained the pressure constant during the whole sequence of injections. Acetonitrile was chosen as the solvent for the final dilution.

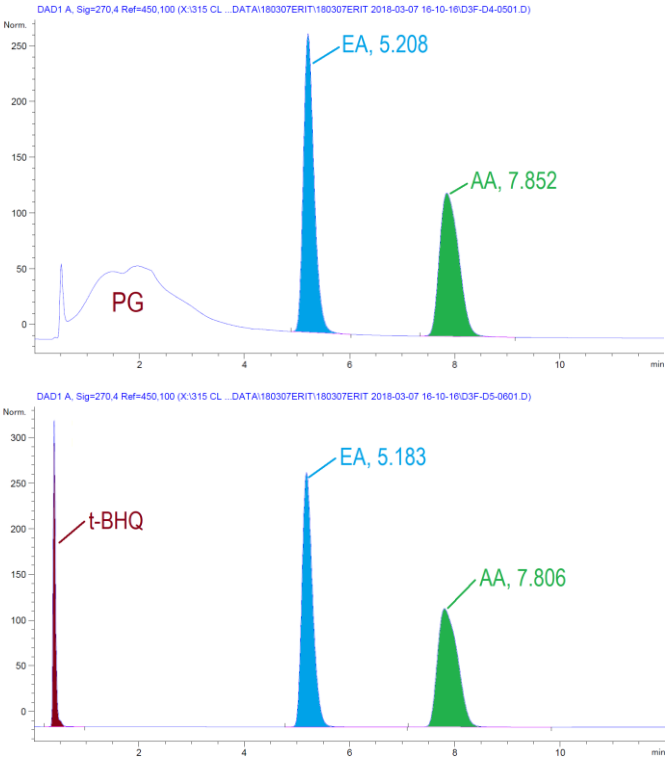

Figure 14: AA and EA HILIC-DAD chromatograms 90:10 acetonitrile-50 mM ammonium acetate aqueous solution, pH 6.5. Aminopropyl column. Dilution of the extract propyl gallate (PG) and tert- butyl hidroquinone (t-BHQ).

In addition, to prevent oxidation during a long sequence of injections, a solution of an antioxidant in acetonitrile was tested. Propyl gallate (PG) and t-BHQ, which are phenolic antioxidants, have been tested. Figure 14 show the chromatograms using 200 mg/L PG acetonitrile solution and 200 mg/L t-BHQ acetonitrile solution to stabilize AA and EA. A distortion of the baseline and a little overlap of PG and EA peaks are observed when PG is used as a stabilizer. Constant pressure is observed using both antioxidants.

A comparative of peak areas diluting with ACN, PG and t-BHQ is shown in Table 5. Peak area values have been recorded in the day of preparation of the standards (1st day) and the next day (2nd day). For each day, the gain of peak area of AA and EA is expressed as recoveries (%). The results obtained using antioxidants are referenced to the results obtained without using a stabilizer (giving a recovery of 100% for each analyte) in the same day of analysis. According

to the results obtained, dilution with t-BHQ acetonitrile solution shows a loss of area lower than acetonitrile and PG for both analytes. Also, as the influence in the first day analysis was not clear, the same standard solutions were analysed the second day after preparation, in order to see whether the antioxidants have a true effect prevent degradation besides PG does not elute correctly in these chromatographic conditions.

The addition of t-BHQ shows better recoveries than the other two options. Though in the preparation day a relevant effect is not clear, at the second day is confirmed a better preservation activity among oxidation.

Table 5: Influence of the addition of PG and t-BHQ to the recoveries of AA and EA.

Stabilizer	Analyte	Peak area (A.U.) 1st day	Peak area (A.U.) 2nd day	Recovery (%) 1st day	Recovery (%) 2nd day
ACN (no stabilizer)	AA	2957.60	2552.47	100	100
	EA	3395.68	2891.15	100	100
PG	AA	2970.23	2670.21	100.4	105.8
	EA	3436.96	3084.15	101.0	106.7
t-BHQ	AA	3100.55	2792.76	104.8	110.7
	EA	3542.36	3155.48	104.3	109.1

Because of preservation activity and peak does not overlap analyte peaks, t-BHQ is chosen as the stabilizer during the final dilution.

6.4. VALIDATION: QUALITY PARAMETERS

6.4.1. Extraction efficiency

Because of degradation of the analytes at small concentrations, recovery experiments were studied for both AA and EA in sausage and chorizo samples spiked at 30 mg/Kg, 50 mg/Kg and 800 mg/Kg. Efficiency of the extraction process has been measured by the recovery percentages of these spiking levels for sausage and chorizo samples. For this method,

recoveries in a range between 85-115% are accepted. As shown in Table 6, AA and EA show recoveries into this range.

Table 6: Average recoveries (%) of the method at the different spiking levels for the analysis for AA and EA.

Analyte	Recovery (%)
AA	95.4
EA	95.8

6.4.2. Linearity

Linearity was assessed in three ranges for both analytes using aqueous solutions of standards, as mentioned in section 5.2.3. In the LASPB, the criteria for acceptable linearity demands correlation coefficients $r \geq 0.995$ at all concentration levels, with an RSD of response factors ≤ 25% for the lowest concentration level and ≤ 15% for the other concentration levels.

A weighting factor of 1/A, where A is peak area, is used for the calibration curves. For unknown samples, three calibration curves must be prepared, as linearity between 6 and 300 mg/L could not be achieved. Calibration curves are prepared daily.

Table 7 shows examples in different days of linearity of both analytes in all ranges established.

Table 7: Linear equation, correlation coefficient (r) and RSD of the response factors (%) of the method.

Analyte	Range	Linear equation	r	RSD of response factors (%)
AA	6-20 mg/L	Y = 71.1x – 222.2	0.9977	26.9
	20-80 mg/L	Y = 82.8x – 695.6	0.9994	18.2
	90-300 mg/L	Y = 94.8 – 1422.7	0.9997	6.2
EA	6-20 mg/L	Y = 83.8x – 162.1	0.9978	14.9
	20-80 mg/L	Y = 86.5x – 503.8	0.9996	11.7
	90-300 mg/L	Y = 97.2 – 1031.1	0.9997	4.4

Acceptable correlation has been seen for all ranges and for both AA and EA, excellent for concentrations higher than 20 mg/L. RSD of response factors decrease when concentration of standards increases as it is expected. A weighting factor of 1/A is used to compensate the low repeatability of response factors to achieve good linearity.

Examples of calibration curves for EA of each level are shown in Figures 15, 16 and 17. AA calibration curves show similar shape comparing to the examples proposed.

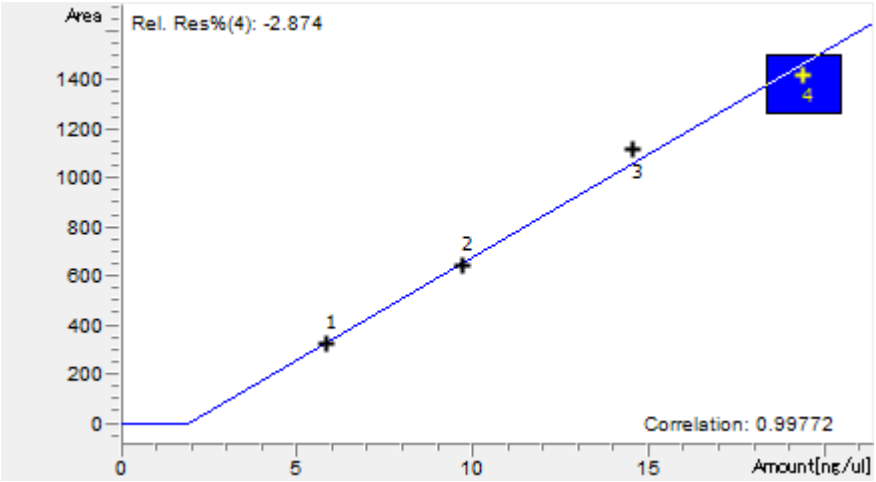

Figure 15: Calibration plot of EA from 6 to 20 mg/L.

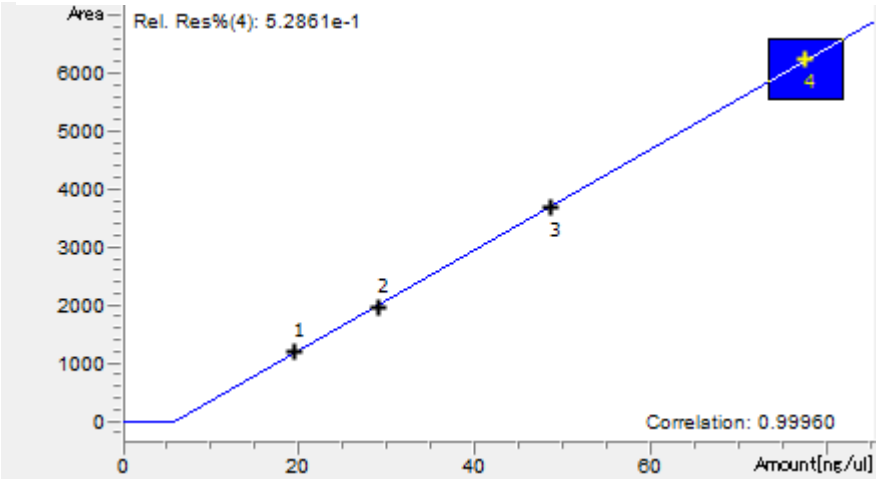

Figure 16: Calibration plot of EA from 20 to 80 mg/L.

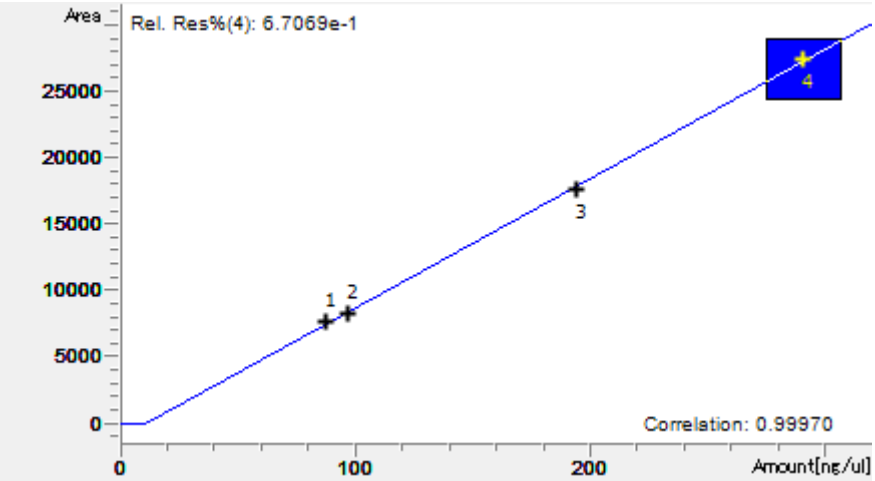

Figure 17: Calibration plot of EA from 90 to 300 mg/L.

6.4.3. Precision and trueness

Precision and trueness are evaluated from intermediate precision and recoveries, respectively, of the spiked samples at the 30 mg/L (LOQ), at 50 mg/L and 800 mg/L in sausage and chorizo samples. Precision is measured by the relative standard deviation (RSD). Replicate experiments (n=12) at each spiking level were performed. Results of the experiments are shown in Table 8. Recovery of AA and EA for both sample types ranges from 94 to 100%, values into the acceptance range (85-100%) established in LASPB.

RSD values have been obtained at different levels range from 4.0 to 8.5%, some of them were higher than the RSD of Horwitz (5.9 %), as in LASPB Horwitz criteria is used as reference. Degradation during the extraction process may be the cause of these high RSD values. These values are accepted as the instability of the analytes.

Table 8: Precision (RSD) and trueness (Recovery) of the method for sausage and chorizo samples at different concentration levels.

Analyte	30 mg/L		50 mg/L		800 mg/L	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
AA	98.2	7.3	97.3	5.0	95.1	8.5
EA	99.8	4.8	96.5	4.0	94.2	5.4

6.4.4. Uncertainty

Uncertainty of the method was determined for AA and EA. The results of recoveries were obtained from both sausage and chorizo samples; at the spiking levels of 30, 50, 100, 500 and 800 mg/L, using the next equation, which is used in LASPB to calculate uncertainty:

$$\mathrm{U} = 2 \cdot \left(\%RSD^2 + \frac{\%RSD^2}{N} + \frac{(100 - \bar{x})}{3} \right)^{1/2}$$

Where U is the uncertainty, %RSD is the RSD of the recoveries at all spiking levels, N is the number of spiked samples (36 samples for both AA and EA) used and $\bar{x}$ is the average of the recoveries of the spiked samples. In LASPB, Horwitz criteria gives the maximum uncertainty permitted to a method validation. A comparison between calculated uncertainty and Horwitz uncertainty is shown in Table 9. The value shown in Table 9 for $U_{Horwitz}$ is calculated for the maximum spiking level (800 mg/Kg).

Table 9: Unertainty (%) of the method for AA and EA for processed-meat samples.

Analyte	$U_{calculated}$	$U_{Horwitz}$
AA	13.4	11.7
EA	12.6	11.7

Uncertainty obtained for both analytes is slightly higher than the Horwitz uncertainty. Normally, this is a case not accepted by LASPB. However, taking into account the instability of AA and EA in aqueous solution, these values are acceptable.

7. CONCLUSIONS

A HILIC-DAD method for the analysis of AA and EA in sausages and chorizo samples has been developed. This method is currently used in Laboratori de l'Agència de Salut Pública de Barcelona (LASPB) for official control analysis.

- An easy extraction process has been developed.
- The chromatographic separation of AA and EA is achieved with an APS-2 Hypersil column (aminopropyl) and a mobile phase consisting on acetonitrile-ammonium acetate 50 mM pH 6.5 aqueous solution (90:10, v/v). UV detection is performed at 270 nm. Total chromatographic run time is 10 min.
- The limit of quantification in samples is 30 mg/Kg.
- Final dilution with a t-BHQ acetonitrile solution lengthen the life of the analytes in the extracts.
- Trueness and precision has been assessed at 30, 50 and 800 mg/L. Trueness, expressed as recovery, ranges from 94-100%. Precision, expressed as %RSD, ranges from 4-8.5%.

Further experiments in the extraction process are necessary to improve stability of AA and EA. A N_2 flow through the standards and extracted solutions could be relevant, removing O_2 present in the solutions. Conserving standards at vacuum or working in an inert atmosphere can be also solutions for the stability problem.

8. REFERENCES AND NOTES

1. André, C.; Castanheira, I.; Cruz, J. M.; Paseiro, P.; Sanches-Silva, A. Analytical strategies to evaluate antioxidants in food: a review. Trends Food Sci. Technol. **2010**, 21, 229-246.
2. Drivelos, S.; Dasenaki, M. E.; Thomaidis, N. S. Determination of isoascorbic acid in fish tissue by hydrophilic interaction liquid chromatography-ultraviolet detection. Anal. Bioanal. Chem. **2010**, 397, 299-2210.
3. Fenoll, J.; Martínez, A.; Hellín, P.; Flores, P. Simultaneous determination of ascorbic and dehydroascorbic acids in vegetables and fruits by liquid chromatography with tandem-mass spectrometry. Food Chemistry. **2011**, 127, 340-344.
4. Tai, A.; Gohda, E. Determination of ascorbic acid and its related compounds in foods and beverages by hydrophilic interaction liquid chromatography. J. Chromatogr. B. **2007**, 853**,** 214–220.
5. Kall, M. A.; Andersen, C. Improved method for simultaneous determination of ascorbic acid and dehydroascorbic acid, isoascorbic acid and dehydroisoascorbic acid in food and biological samples. J. Chromatogr. B. **1999**, 730, 101-111.
6. Barros, A. I. R. N. A.; Silva, A. P.; Gonçalves, B.; Nunes, F. M. A fast, simple, and reliable hydrophilic interaction liquid chromatography method for the determination of ascorbic and isoascorbic acids. Anal. Bioanal. Chem. **2010**, 396, 1863-1875.
7. Scientific Opinion on the re-evaluation of ascorbic acid (E 300), sodium ascorbate (E 301) and calcium ascorbate (E 302) as food additives. EFSA Journal, **2015**, 13(5):4087, 1-124.
8. Scientific Opinion on the re-evaluation of erythorbic acid (E 315) and sodium erythorbate (E 316) as food additives. EFSA Journal, **2016**. 14(1):4360, 1-51.
9. Versari, A.; Mattioli, A.; Parpinello, G. P.; Galassi, S. Rapid analysis of ascorbic and isoascorbic acids in fruit juice by capillary electrophoresis. Food Control. **2004**, 15, 355–358.
10. Thompson, C. O.; Trenerry, V. C. A rapid method for the determination of total L-ascorbic acid in fruits and vegetables by micellar electrokinetic capillary chromatography. Food Chemistry. **1995**, 53, 43-50.
11. Valette, J. C.; Demesmay, C.; Rocca, J. L.; Verdon, E. Separation of tetracycline antibiotics by hydrophilic interaction chromatography using an amino-propyl stationary phase. Chromatographia. **2004**, 59, 55-60.
12. Douša, M. 1H-Tetrazole-5-amine immobilized on substituted polymer gel/silica as a new stationary phase for hydrophilic interaction chromatography. Chromatographia. **2017**, https://doi.org/10.1007/s10337-017-3452-6
13. Alpert, A. J. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. J. Chromatogr. A. **1990**, 499, 177-196
14. Buszewski, B.; Noga, S. Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. Anal. Bionala. Chem. **2012**, 402, 231-247

9. ACRONYMS

AA: Ascorbic acid

AA-2BG: 2-O-B-D-glucopyranosyl L-ascorbic acid

AA-2G: 2-O-Glucopyranosyl L-ascorbic acid

ACN: Acetonitrile

ADI: Acceptable daily intake

DAD: Diode array

DHAA: Dehydroascorbic acid

DHEA: Dehydroerythorbic acid

DKG: 2,3-diketo-L-gulonic acid

DKGluA: 2,3-diketogluconic acid

DTT: Dithiotreitol

EA: Erythorbic acid

EDTA: Etilendiaminetetraacetic acid

EU: European Union

HILIC: Hydrophilic interaction liquid chromatography

LASPB: Laboratori de l'Agència de Salut Pública de Barcelona

LC: Liquid chromatography

LOQ: Limit of quantification

MPA: m-Phosphoric acid

MRL: Maximum residue levels

RP-LC: Reversed phase liquid chromatography

t-BHQ: tert-Butylhydroquinone

TCEP: Tris-2-carboxyethylphosphine

TFA: Trifluoroacetic acid

UHPLC: Ultra high-performance liquid chromatography

UV: Ultraviolet spectroscopy

APPENDICES

APPENDIX 1: ALLOWANCE OF ASOCRBIC ACID AS FOOD ADDITIVE

Table 10 shows the allowance of AA as a food additive in different meat food samples, which are related to the food samples analysed in this study.

Table 10: Allowance of AA as food additive provided by FCS (Food Categorization System) presented in Annex II to Regulation (EC) No 1333/2008

FCS category number	FCS food category	E-number	Name	MPL (mg/L or mg/kg as appropriate)	Restrictions / exceptions
07.2	Fine bakery wares	Group I	Additives	*Quantum satis*	
08.2	Meat preparations as defined by Regulation (EC) No 853/2004	E 300/E 301/E 302	Ascorbic acid/sodium ascorbate/calcium ascorbate	*Quantum satis*	Only gehakt and prepacked preparations of fresh minced meat
08.3.1	Non-heat-treated meat products	Group I	Additives	*Quantum satis*	
08.3.2	Heat-treated meat products	Group I	Additives	*Quantum satis*	Except *foie gras, foie gras entier, blocs de foie gras, libamaj, libamáj egészben, libamáj tömbben*
08.3.2	Heat-treated meat products	E 300/E 301	Ascorbic acid/sodium ascorbate	*Quantum satis*	
08.3.3	Casings and coatings and decorations for meat	Group I	Additives	*Quantum satis*	

09.1.1	Unprocessed fish	E 300/E 301/E 302	Ascorbic acid/sodium ascorbate/calcium ascorbate	*Quantum satis*	
09.1.1	Unprocessed molluscs and crustaceans	E 300/E 301/E 302	Ascorbic acid/sodium ascorbate/calcium ascorbate	*Quantum satis*	
09.2	Processed fish and fishery products including molluscs and crustaceans	Group I	Additives	*Quantum satis*	
09.3	Fish roe	Group I	Additives	*Quantum satis*	Only processed fish roe

APPENDIX 2: ALLOWANCE OF ERYTHORBIC ACID AS FOOD ADDITIVE

Table 11: Allowance of EA as food additive provided by FCS (Food Categorization System) presented in Annex II to Regulation (EC) Nº 1333/2008

FCS category number	FCS food category description	E-number	Restrictions/exceptions	MPL (mg/L or mg/kg as appropiate)
08.3.1	Non-heat-treated meat products	E 315	Only cured meat products and preserved meat products	500 (a)
		E 316	Only cured meat products and preserved meat products	500 (a)
08.3.2	Heat-treated meat products	E 315	Only cured meat products and preserved meat products	500 (a)
		E 316	Only cured meat products and preserved meat products	500 (a)
09.1.1	Unprocessed fish	E 315	Only frozen and deep-frozen fish with red skin	1500 (a)
		E 316	Only frozen and deep-frozen fish with red skin	1500 (a)
09.2	Processed fish and fishery products including molluscs and crustaceans	E 315	Only frozen and deep-frozen fish with red skin	1500 (a)
		E 316	Only frozen and deep-frozen fish with red skin	1500 (a)
09.3	Fish roe	E 315	Only frozen and deep-frozen fish with red skin	1500 (a)
		E 316	Only frozen and deep-frozen fish with red skin	1500 (a)

(a) E315 Erythorbic acid and E316 sodium erythorbate are authorised individually or in combination, MPL (maximum permitted level) is expressed as erythorbic acid.