U.PORTO

MESTRADO

TECNOLOGIA FARMACÊUTICA

# Comparative study of ascorbic acid and derivatives with interest in anti-aging cosmetics

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2017

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# Comparative study of ascorbic acid and derivatives with interest in anti-aging cosmetics

Master in Pharmaceutical Technology

Work carried out under the supervision of Professor Isabel Almeida and co-supervision of Professor Emília Sousa

Porto, September 2017

(Assinatura do autor)

**Author's declaration:**

Under the terms of the Decree-Law nº 216/92, of October 13th, is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work and interpretation of the results included in this dissertation.

Under the terms of the referred Decree-Law, is hereby declared that the following articles/communications were prepared in the scope of this dissertation. The results presented in this dissertation are part of the following scientific works:

***Article in preparation:***

Sílvia Silva, Ana S. Oliveira, Catarina Magalhães, Maria E. Sousa, Madalena M. M. Pinto, José M. Sousa-Lobo, Isabel F. Almeida. Trends in the use of antioxidants in anti-aging cosmetics. (in preparation)

***Poster communications***

S Silva, A Oliveira, C Magalhães, ME Sousa, JM Sousa Lobo, IF Almeida. Trends in use of antioxidants in anti-aging cosmetics. Skin Forum, London, UK, 23 of June 2016.

**Acknowledgments**

No final desta dissertação, marco de uma aventurosa viagem académica, pretendo agradecer às pessoas cuja vontade convergiu na concretização do meu projecto. Por isso, a todas as pessoas, que de algum modo me auxiliaram neste itinerário, que fique expresso o meu reconhecimento e gratidão.

De modo particular dirijo-me com votos de agradecimento à minha orientadora, a Professora Doutora Isabel Almeida, pela sua colaboração, compreensão, disponibilidade e auxílio ao construir críticas, opiniões, vias e soluções que foram fundamentais para o encaminhamento deste trabalho. Agradeço a sua ambição neste projeto que me levou a desenvolver-me em diversas vertentes bem como em participar em atividades que me fizeram crescer enquanto profissional. À minha co-orientadora Professora Doutora Emília Sousa agradeço o apoio incondicional no encaminhamento das tarefas experimentais e pelo ânimo nas situações de angústia e cansaço.

Igualmente, ao Professor Doutor Domingos Ferreira, que acompanhou todo o meu trajeto no mestrado em tecnologia farmacêutica; ao Professor Doutor Paulo Costa que pela sua destreza na operação com os equipamentos e pelo auxílio quando mais necessitava. À Professora Doutora Helena Amaral pela compressão demonstrada no decorrer dos trabalhos.

Expresso igualmente uma mensagem de gratulação a todo o núcleo de professores do departamento que de alguma forma, pela mais simples ajuda, permitiram que hoje tenha o meu trabalho concluído. À D. Conceição e restantes funcionários do departamento que tornam possível o desenvolvimento da parte experimental. À Dra. Sara Cravo e à Gisela Adriano, pela assistência nas técnicas laboratoriais e pelo fornecimento de conhecimentos práticos durante o período da realização da dissertação.

Ao meu namorado João Santos, pelo apoio e pelos bons momentos…

Às minhas amizades, da terra, que me desanuviaram nos tempos mais conturbados, Salomé, Ranzal, André, Vítor, Dani; e, às do departamento, e em especial, um agradecimento à Ana Daniela que se tornou de uma forma espontânea a pessoa mais peculiar para mim com palavras de apoio e vontade de entreajuda.

Aos meus colegas de trabalho referindo-me com toda a gratidão à minha colega de trabalho, Sara Silva, pelos momentos de desabafo e encorajamento. À restante equipa, Patrícia, Cristiano e Marisa, que apreciaram o meu gosto pelo encaminhamento dos

meus estudos. Agora, ao Bruno Rocha e à Doutora Eva Pereira, meus patrões, que me presentearam com a sua confiança, disponibilidade, flexibilidade e presunção neste projeto e enquanto colaboradora da então farmácia.

Agradeço ao meu irmão, Miguel Silva, pelo apoio especial demonstrado ao longo destes anos, principalmente nos momentos dos apertos em que nada nos parece ajudar. Á Sónia e à minha pequenina afilhada que pela sua ingenuidade, simplicidade, e imensas brincadeiras, me encoraja e me dá vontade de fazer mais e melhor.

Ao meu pai que pelas palavras enlaçadas e marcadas pela subtilidade e espontaneidade, que me ajudou a tornar o sonho possível e me deu ao mundo numa forma mais feliz, Adão Silva. À minha mãe que me deu ao mundo, Alzira Filipe, e que mesmo não confiando nesta minha opção, hoje, acredite e fique feliz por mais uma etapa cumprida.

A todos que fazem parte do meu dia-a-dia e me fazem feliz 😃!

A todos um bem-haja.

**Inscription**

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*Ao meu avô que partiu na imensidão do*

*seu partir e me deixou a contar as ondas*

*Até não haver mais mar seu para contar.*

*Prendo-me com nostalgia, num tom muito denso de saudades, das suas mãos molhadas de palavras.*

*Ao seu último olhar que me traçou a alma das suas convicções e que me fazem acreditar que é possível…*

*À minha afilhada que por mil fantasias do seu olhar arrasta desenfreadamente a luz que brilha à sua passagem… o seu sorriso.*

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## Abbreviations

3OAA
: 3-O-Ethyl-L-ascorbic acid

AA
: Ascorbic acid

AA2G
: Ascorbic acid 2-O-α-glucoside

AA2GS
: Sulphate ascorbic acid-2-glucoside

ADME
: Absorption, distribution, metabolism and excretion

AEE
: Stearyl alcohol

AP
: Ascorbyl-6-palmitate

CP
: Carbopol

DHA
: Dehydroascorbic acid

DNA
: Deoxyribonucleic acid

DPPH
: 2,2-diphenyl-1-picrylhydrazyl

DSC
: Differential Scanning Calorimeter

FTIR
: Fourrier transformed infrared spectroscopy

GSH
: Glutathione

HEC
: Hydroxyethyl cellulose

IL
: Interleukin

LAA
: L-ascorbic acid

MAP
: Magnesium ascorbyl-2-phosphate

MIG
: Miglyol 812

NFkB
: Nuclear transcription factor kappa B,

NMF
: Natural moisturizing factor

PCP
: Potassium cetyl phosphate

ROS
: Reactive oxygen species

SAP
: Sodium ascorbyl-2-phosphate

SDA
: Semydehydroascorbic acid

T
: Temperature

TNF-α
: Tumour Necrosis Factor α

TP
: Tocopherol

UV
: Ultraviolet light

Vis
: Visible

ΔH
: Enthalpy

## Abstract

Skin health and beauty are cornerstones of general well-being in humans. Anti-aging cosmetics are used to provide a healthy and youthful appearance. Among different cosmetic actives, antioxidants have been incorporated in anti-aging products. Ascorbic acid and its derivatives are being consistently the most used antioxidants in anti-aging formulations.

Ascorbic acid possesses a variety of cutaneous benefits, besides antioxidant activity, including photoprotection from UVA and B radiation, neocollagenesis, inhibition of melanogenesis and improvement of a variety of inflammatory skin disorders. One of the main challenges with using vitamin C in pharmaceutical and cosmetic formulations is its chemical instability, which fostered the development of derivatives with higher stability.

The main aims of this dissertation were to investigate the solubility in water and glycerol, at different pH values (2, 3, 4, 5, 6, 7, 8), the thermal stability (4ºC, 25ºC, 40ºC, 60ºC), the stability in presence of metal ions ($Ca^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Cu^{2+}$) and the photostability of ascorbic acid (AA) and five derivatives commercially available - ascorbyl palmitate, magnesium ascorbyl phosphate, sodium ascorbyl phosphate, 3-*O*-ethyl ascorbic acid, ascorbic acid-2-glucoside – and one new derivative - sulphate ascorbic acid-2-glucoside. The compatibility of these antioxidants with common excipients of topical formulations (stearilic alcohol, carbopol, hydroxyethylcellulose, tocopherol, potassium cetyl phosphate, EDTA and miglyol) was evaluated with DSC. Additionally, their antioxidant activity was also compared by the DPPH reduction assay.

The stability of ascorbic acid and its derivatives studied in dependence on the pH of their aqueous solution revealed that at pH 5 the majority of antioxidants are stable. The derivatives of AA demonstrated to be more stable than AA in the range investigated. Exposure to high temperatures revealed that AA derivatives were also relatively stable. Preferentially, the storage temperatures recommended are 4ºC and 25ºC.

In the presence of $Mg^{2+}$, AA does not suffer any decrease in its concentration, suggesting that magnesium ions do not affect AA stability. The stability of MAP, SAP and 3OAA against $Fe^{2+}$ and $Fe^{3+}$ ion was shown to be high with 90% of their concentration maintained even after 24 h in 100 µM of $Fe^{2+}$ and $Fe^{3+}$ ion, whereas AA was almost completely degraded under these conditions. Likewise, compound 3OAA was more stable

than AA and MAP and SAP, although 3OAA stability was relatively lower in the presence of $Fe^{3+}$ ion.

The results of photostability evaluation showed that, after 15 min of UV exposure, a 40% decrease of AA absorption was noted and AP suffers bathochromic alterations in spectrum. The other antioxidants showed better stability than AA. With respect to thermal analysis of AA, AP, MAP, SAP and AA2G one exothermic peak was detected. In case of AA2GS an endothermic peak was observed. After analysing the thermograms from binary mixtures, strong interactions between the antioxidants and EDTA, and PCP can be assumed because the active ingredient melting peak almost disappeared and/or $T_{peak}$ is not possible to be precisely determined. AEE, CP, EDTA, PCP, TP, MIG, HEC it can be assumed that some excipients did not affect some antioxidants thermal behaviour without any apparent physical-chemical change or incompatibility. The antioxidants with higher compatibility with the tested excipients were SAP, AA2G and AA2GS. AP and 3OAA was the antioxidant with more incompatibilities.

The highest antioxidant activity, as evaluated with DPPH reduction assay, was obtained for AA followed by 3OAA, SAP, AA2G and AA2GS.

Overall, the most stable derivative of AA was found to be 3OAA. The new compound has a similar behaviour as its parent AA2G and is highlighted by its highest compatibility with a variety of typical excipients of semi-solid formulations.

**Keywords**: Ascorbic acid, ascorbic acid derivatives, stability, antioxidants, cosmetics

## Resumo

A saúde da pele e a sua beleza são pedras angulares no bem-estar geral dos humanos. Os cosméticos anti-envelhecimento são utilizados de forma a fornecer uma aparência saudável e jovem. Entre os diferentes ingredientes activos dos cosméticos, os antioxidantes têm sido incorporados nos produtos anti-idade. Topicamente aplicados os antioxidantes exercem os seus benefícios ao oferecer protecção contra os radicais livres. Os demais produtos cosméticos incorporam antioxidantes são os mais populares produtos anti-idade. O ácido ascórbico e os seus derivados têm sido consistentemente os antioxidantes mais utilizados nas formulações anti-idade. Os resultados de um estudo prévio demonstraram que de entre os demais antioxidantes, durante os últimos cinco anos, o ácido ascórbico e os seus derivados são amplamente utilizados. Em 2015, dois derivados incluídos nesta dissertação, o ácido ascórbico glicosilado e o palmitate de ascorbilo apareciam no "*top ten*" dos antioxidantes mais utilizados. Em 2011 e 2013, o ácido ascórbico e os seus derivados – o fosfato de magnésio e fosfato de sódio do ácido ascórbico permaneceram no "*top ten*".

O ácido ascórbico tem sido amplamente utilizado, por possuir uma variedade de benefícios cutâneos, além da sua actividade antioxidante, incluindo a fotoproteção contra a radiação UVA e B, a promoção da neocolagénese, a inibição da melanogénese e a melhoria de uma variedade de desordens inflamatórias da pele. Um dos principais desafios com o uso de vitamina C em formulações farmacêuticas e cosméticas é sua instabilidade química, que promoveu o desenvolvimento de derivados com maior estabilidade.

Os objectivos principais desta dissertação foram investigar a solubilidade em água e glicerina, em valores de pH diferentes (2, 3, 4, 5, 6, 7, 8), a estabilidade térmica (4ºC, 25ºC, 40ºC, 60ºC), a estabilidade na presença de iões metálicos ($Ca^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Cu^{2+}$) e a fotoestabilidade do ácido ascórbico (AA) e de cinco derivados disponíveis comercialmente - palmitato de ascorbilo, fosfato de ascorbilo e magnésio, fosfato de ascorbilo e sódio , ácido etil-3-*O*- ascórbico, ácido ascórbico-2-glicosídeo – e um novo derivado – o sulfato do ácido ascórbico-2-glicosídeo. A compatibilidade destes antioxidantes com excipientes comuns em formulações tópicas (álcool estearílico, carbopol, hidroxietilcelulose, tocoferol, fosfato de potássio cetílico, EDTA e miglyol) foi

avaliada por DSC. Além disso, a sua actividade antioxidante foi também comparada pelo ensaio de redução do DPPH.

A estabilidade do ácido ascórbico e seus derivados foi estudada em relação ao pH das suas soluções aquosas e revelou-se que, a pH 5, a maioria dos antioxidantes é estável. Os derivados de AA demonstraram ser mais estáveis do que AA na faixa de pH investigados. A exposição a temperaturas elevadas revelou que os derivados de AA também eram relativamente estáveis. Preferencialmente, as temperaturas de armazenamento recomendadas são 4ºC e 25ºC.

Na presença de $Mg^{2+}$, o AA não sofre qualquer diminuição da sua concentração, sugerindo que os iões de magnésio não afectam a estabilidade do AA. A estabilidade de compostos MAP, SAP e 3OAA na presença de $Fe^{2+}$ e $Fe^{3+}$ é elevada com 90% da sua concentração mantida mesmo após 24 h em 100 µM iões $Fe^{2+}$ e $Fe^{3+}$, enquanto o AA foi quase completamente degradado nestas condições. O composto 3OAA demonstrou ser mais estável que o AA, o MAP e o SAP, embora a sua estabilidade ter sido relativamente menor na presença de iões de $Fe^{3+}$.

Os resultados da avaliação da fotoestabilidade mostraram que, após 15 min de exposição aos raios UV uma diminuição de 40% da absorção de AA e, por seu turno, o AP sofre alterações batocrómicas no espectro. Os outros antioxidantes mostraram melhor estabilidade do que AA. No que diz respeito a análise térmica de AA, AP, MAP, SAP e AA2G apresentam um pico exotérmico. No caso de AA2GS, observou-se um pico endotérmico. Depois de analisar os termogramas de misturas binárias, com AEE, CP, EDTA, PCP, TP, MIG, HEC, pode-se supor que estes excipientes não afectam o comportamento térmico dos antioxidantes não tendo sido observado qualquer mudança físico-química aparente ou incompatibilidade. Os antioxidantes com maior compatibilidade com os excipientes estadas foram AA2G e AA2GS.

A actividade antioxidante mais elevada, conforme avaliado com ensaio de redução de DPPH, obteve-se para AA seguido por 3OAA, SAP e AA2G e AA2GS.

No geral, o derivado mais estável da AA é o 3OAA. O novo composto (AA2GS) apresenta um comportamento semelhante ao seu análogo AA2G e é destacado pela sua elevada compatibilidade com os excipientes utilizados nas preparações semi-sólidas.

**Palavras-chave**: Ácido ascórbico, derivados do ácido ascórbico, estabilidade, antioxidantes, cosméticos.

## Outline of this dissertation

CHAPTER 1 – INTRODUCTION

Chapter 1 includes a brief introduction to ascorbic acid and its derivatives as anti-aging active ingredients with interest in formulation of cosmetics. Their physical and chemical properties, pharmaceutical and technologic aspects are also presented.

CHAPTER 2 – AIMS

In this chapter, the main objectives are described.

CHAPTER 3 - MATERIAL AND METHODS

In this chapter, the general methods including the materials used, reagents and also characterization methods are described as well as the experimental procedures and conditions used in each study.

CHAPTER 4 - RESULTS AND DISCUSSION

Results and discussion are divided into four topics. The first topic describes the studies towards the solubility in water and glycerol. The second topic concerns the chemical stability of all compounds. This topic is also subdivided in evaluation of pH stability, thermal stability, and stability in presence of metals and photostability. The third topic deals with the compatibility of compounds with broadly employed excipients in cosmetic formulations. Finally, the fourth topic describes the results of the antioxidant capacity of each investigated compound.

CHAPTER 5 – CONCLUSIONS

This chapter summarizes the main conclusions concerning this dissertation.

CHAPTER 6 – REFERENCES

In this chapter, the references cited throughout the dissertation are presented. The main bibliographic search engines/databases were ISI Web of Knowledge, Scopus, PubMed and Google Scholar.

# Chapter I: Introduction

## Chapter I: Introduction

Aging is defined by some authors as an universal progressive irreversible intrinsic process which all living things suffer from, as an expression of the interaction between genetics of the individual and their environment aggressors (Reynolds D. *et al*, 2002; Murkheerjee P. *et al*., 2011). Avoid aging has been one of the greatest ambitions of human beings and, consequently, to combat aging is a challenge for modern medicine. In modern society, there is a great increase in the search for eternal youth (Silva, JA *et al.* 2010; Murkheerjee P. *et al*., 2011). Over time, most of the functions of the various organs and tissues in the body decrease their activity, either by alterations in cellular metabolic activity or by processes that affect these cells. With the aging associated with the chronological age, a proteolytic degradation of the fiber network at a cellular level occurs, which leaves visible signs on the surface of the skin (Murkheerjee P. *et al*., 2011). All individual systems exhibit this process, but each one develops differently, what characterizes old age of great biological variability (Reynolds D. *et al*, 2002; Murkheerjee P. *et al*., 2011).

There are many cosmetic ingredients that are claimed to have anti-aging effects when used topically. These products are geared in the prevention and treatment of broad term anti-aging, which derives mainly from photoaging that is characterized by clinical signs including irregular dryness, dark/light pigmentation, sallowness, deep furrows or severe atrophy, telangiectases, premalignant lesions, laxity, and a leathery appearance (Burke K, 2004; Silva JA *et al.* 2010). Other signs include elastosis (a coarse, yellow, cobblestoned effect of the skin) and actinic purpura (easy bruising related to vascular wall fragility in the dermis) (Bissett D., 2005). Therefore, the anti-aging cosmetics are based on the prevention of these signs in support of improving the wide range of signals, in which they can bring benefits such as wrinkles, sagging, texture, pallor, hyperpigmentation, etc. (Bissett D., 2005; Burke K, 2004; Murkheerjee P. *et al*., 2011; Silva, JA *et al.* 2010).

Burke *et al*. (2006) defends that topical application of antioxidants can give far higher concentrations in the skin than maximal oral intake. However, the correct formulation is of utmost importance to attain efficacy. The challenge is to use the correct form of the antioxidant molecule, to keep the antioxidant active to attain a reasonable shelf-life for the product, and to achieve effective transcutaneous absorption that delivers effectively high concentrations of the active antioxidant to the dermis as well as the epidermis.

The results of a previous study showed that among antioxidants, over five years AA and derivatives are widely used. In 2015, two derivatives included in this dissertation, ascorbyl glucoside and ascorbyl palmitate reached the top ten antioxidants. In 2011 and 2013, ascorbic acid and its derivatives: magnesium and sodium ascorbyl phosphate reached the top ten. For this reason it is compiled in this dissertation a comparative study of ascorbic acid and derivatives: magnesium ascorbyl phosphate (MAP), ascorbyl palmitate (AP), sodium ascorbyl phosphate (SAP), ascorbic acid-2-glucoside (AA2G), sulphate ascorbic acid-2-glucoside (AA2GS) and 3-*O*-ethyl ascorbic acid (3OAA) to analyse the best physical and chemical characteristics that justifies a widely use in anti-aging cosmetics.

## Vitamin C

### *Discovery*

Following the outbreak of scurvy during World War I, it was shown that germinating, but not dry, cereals and legumes are effective against scurvy in monkeys and guinea pigs (Bissett DL, 2005). Although, it was not known which compound present in these fresh foods reduced the expression of scurvy. However, people linked the appearance of scorbutus with an insufficient ingestion of vegetables and fruits. Like this, scorbutus was common in discoveries epoch and in greatest wars where food resources are limited (Nakamura S, 2009; Takebayashi J *et al.* 2006; Santos M, 2014). In 1928, Albert Szent-Gyorgyi isolated a six-carbon reducing substance from oranges and cabbages. In 1932, he and C.C. King showed this substance to be the antiscorbutic principle when identifying its role in scurvy (Cadenas E, Packer L., 2007; Telang P, 2013). Albert Szent-Gyorgyi named it ascorbic acid (AA) and was awarded the Nobel Prize in 1937 (Bissett DL, 2005; Cadenas E, Packer L., 2007).

### *Sources*

Most plants and animals can synthesise ascorbic acid (AA), *in vivo* from glucose. Animals synthesize AA from glucose in the liver (in mammals) or kidneys (birds and reptiles). Several species of animals, distributed through the evolutionary tree, are unable to synthetize vitamin C. These include human and nonhuman primates, guinea pigs, indian fruit bats, bulbus, and some fish (Cadenas E, Packer L., 2007; Pinnell SR, 2003).

In the human, the gene encoding gulonolactone oxidase enzyme has extensive mutations, so that there is no protein product (Cadenas E, Packer L., 2007; Pinnell SR, 2003). Humans and animal's incapable to synthetize vitamin C usually obtain from exogenous sources sufficient amounts from their largely plant diet acquired from citrus fruit, green leafy vegetables, strawberries, papaya and broccoli where this antioxidant is present in its reduced form: L-ascorbic acid (LAA) and in its oxidation product dehydroascorbic acid (DHA), both with vitamin activity (Telang P, 2013, Santos M, 2014). Vitamin C is synthetized by plants from several precursors and is abundant in leaves, and, in particular, the chloroplast. It may play a role in photosynthesis, stress resistance, and plant growth and development (Cadenas E, Packer L., 2007).

### *Vitamin C: physical and chemical properties*

AA is a natural water-soluble vitamin (Stamford NP, 2012). This non-enzymatic antioxidant acts by scavenging and reducing reactive species to less reactive species (type 2 mechanism) (Telang P, 2013; Takebayashi J *et al.* 2002).

AA corresponding to a ketolactone with 6 carbons and the molecular formula $C_6H_8O_6$ (Figure 1) is very unstable and easily oxidized in aqueous solutions and cosmetic formulations. It can be used with α-tocopherol acting as a co-antioxidant with a synergic effect (Telang P, 2013; Santos M, 2014).

Hence, vitamin C or ascorbic acid or even ascorbate is a low molecular weight six-carbon lactone with hydrophilic, acidity and power reducing character (Cadenas E, Packer L., 2007; Pinnell SR, 2013, Telang P, 2013; Santos M, 2014; Oroian *et al.* 2015). Table 1 summarizes and highlights the physical and chemical properties of AA.

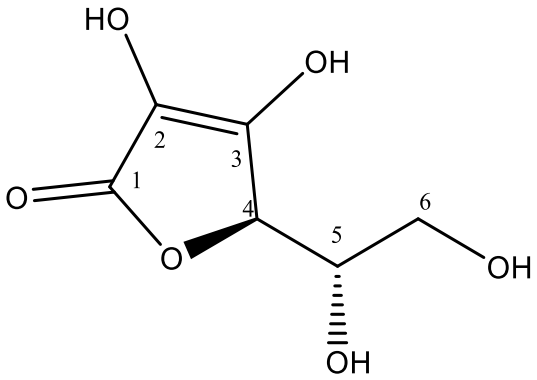


**Figure 1** Ascorbic acid: structural formula.

**Table 1** Physical and chemical properties of ascorbic acid (Rowe *et al.* 2009).

| Property | | Ascorbic acid specifications |
|---|---|---|
| **IUPAC name** | | (2*R*)-2-[(1*S*)-1,2-dihydroxyethyl]z-3,4-dihydroxy-2*H*-furan-5-one |
| **Appearance** | | White, odourless, crystalline solid with sharp acidic state |
| **Formula** | | $C_6H_8O_6$ |
| **Molecular mass** | | 176.13 |
| **Density** | | 1.65 g/cm$^3$ |
| **pH** | | ≈ 3 |
| **pKa** | | $pKa_1$=4.17<br>$pKa_2$=11.57 |
| **Solubility** (g/ml) | *Water* | 0.33 |
| | *Glycerol (USP)* | 0.01 |
| | *Fats and oils solvents* | Insoluble |

As mentioned above, AA has a 5-hydrocarbon ring similar to that of glucose. If in the ascorbic acid a hydrogen ion is associated, L-ascorbic acid (LAA) becomes a weak sugar acid, similar to other α-hydroxyacids used in dermatology (Davey M *et al.* 2000). With a metal ion, it forms a mineral ascorbate (Telang P, 2013; Takebayashi J, 2014).

In solution, delocalisation of the π-electrons over the C2-C3 conjugated enediol system stabilises the molecule and causes the hydrogen of the C3 hydroxyl to become highly acidic, and to dissociate with a pKa of 4.13. AA is stable at a pH values below the first pKa. At physiological pH, LAA exists as a monovalent anion (L-ascorbate). Dissociation of the second hydroxyl takes place at pH 11.6 (Cadenas E, Packer L., 2007; Telang P, 2013; Takebayashi J, 2014; Austria R. *et al.* 1997).

L-Ascorbic acid is the chemically active form of vitamin C. In nature, ascorbic acid is found in equal parts as L-ascorbic acid (LAA) and D-ascorbic acid (DHA). These are essentially isomeric molecules and are mutually interchangeable as mentioned previously. Nevertheless, only LAA is biologically active and thus useful in pharmaceutical and cosmetic formulations (Humbert PG *et al*. 2003; Telang P, 2013; Shalmashi A *et al*. 2008; Hacisekvi A, 2009; Sheraz M *et al.* 2011). AA in powder is relatively stable in air. In the absence of oxygen and other oxidizing agents it is also heat stable (Rowe R *et al.* 2009).

AA oxidation is generated by its ionization in aqueous solution (Austria R. *et al.* 1997; Stamford NP, 2012). In solution, AA readily undergoes oxidation; however, other factors increase the rate of degradation of vitamin C particularly alkaline solution (pH>7), Ultraviolet light (UV) exposure and heat, metal ions in amounts that catalyse its oxidation (e.g. traces of copper and iron and other oxidants) and presence of dissolved oxygen (Tipson R, Horton D, 1997; Davey M *et al.* 2000; Stamford NP, 2012). Ascorbic acid solutions exhibit maximum stability at about pH 5.4 (Rowe R *et al.* 2009; Stamford NP, 2012).

This vitamin is usually used at 1 to 20% of concentration in topical formulations containing an acidic pH (around 3.5) to effectively penetrate the skin (Sheraz M *et al.* 2011). Vitamin C is extremely reactive and unstable in dispersions due to its fast oxidation and further irreversible chemical transformation. Indeed, the degradation by oxidation of AA most often accompanied by a yellowish discolouration (Shalmashi A *et al*. 2008; Stamford NP, 2012).

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### *Ascorbic acid biological functions and metabolism*

Besides its involvement in metabolic functions such as fighting bacterial infections, detoxifying reactions and formation of collagen, AA exerts several functions on the skin such as collagen synthesis, depigmentation and antioxidant activity, being widely used in anti-aging skin care formulations (Sheraz M *et al*. 2011; Silva GM, Maia Campos PM, 2000; Smart RC, Crawford CL, 1991; Stojiljkovic D *et al*. 2014).

AA is a cofactor for numerous enzymes participating in the post-translational hydroxylation of collagen, in the production of L-carnitine, in bioconversion of some neurotransmitters, and tyrosine metabolism (Hacisekvi A, 2009; Oresajo C *et al*. 2012; Pandel R *et al*. 2013; Pillai S *et al*. 2005; Pinnell SR, 2003).

AA has three main types of distinct biological activities in animals as an enzyme co-factor; as a direct physiological radical scavenger and, finally as a donor/acceptor in electron transport in plasma membrane (Masaki H, 2010; Mukherjee PK et al. 2011; Oresajo C *et al*. 2012; Smart RC, Crawford CL, 1991). In this manner, AA is a water soluble radical scavenger widely distributed in aerobic organisms that plays an essential role in defence of cellular components against oxidative damage by free radicals and oxidants that are involved in the progress of a chronic diseases such as cancer, brain dysfunction, aging (Masaki H, 2010; Pinnell SR, 2003), heart disease (Lynch SM et al. 1996), inflammation, and stroke (Telang P, 2013). Ascorbate is an electron donor, and this property accounts for its known and postulated functions (Sies H, 1997; Cadenas E, Packer L, 2007; Poljak B et al. 2011; Telang P, 2013). It is believed that AA is effective in scavenging superoxide radical anions, hydroxyl radicals, hydrogen peroxide, reactive nitrogen species and singlet oxygen (Oroian M et al. 2015). AA has, at a structural level, 4 hydroxyl groups which can donate hydrogen to an oxidizing system (Oroian M *et al*. 2015). So, AA has its redox potential are strictly associated with the electron rich C2-C3-enediol moiety of its five-membered lactone ring (Olabisi A, 2004). When it donates two electrons from mentioned bonds, the intermediate free radical semydehydroascorbic acid (SDA) – ascorbate free radical is formed - Figure 2 (Olabisi A, 2004; Nimse S, Pal D, 2015).

The ascorbate free radical is unstable, but is much less reactive than other free radicals provided by other compounds that have potential to form harmful free radicals (Cadenas E, Packer L, 2007; Oroian M *et al.* 2015). Besides, SDA can be reversibly reduced to ascorbate (Oroian M *et al*. 2015). These properties make AA an ideal

electron donor (Poljak B *et al*. 2011; Puvabanditsin P, Vongtongsri R, 2016; Stojiljkovic D *et al*. 2014).

SDA, being unstable, undergoes further oxidation to form the more stable product, DHA which can exist in more than one structural form, but only a few minutes at physiological pH (Cadenas E, Packer L, 2007; Nimse S, Pal D, 2015).

DHA can be reduced back to ascorbate by enzyme dehydroascorbic acid reductase in the presence of glutathione (GSH) (Pinnell SR, 2003; Telang P, 2013), with formation of glutathione disulphide or by enzymatic reduction (Cadenas E, Packer L, 2007). If not reduced, DHA decay as the lactone ring irreversibly opens and it is hydrolysed to 2,3-diketogulonic acid (Hacisekvi A, 2009; Humbert PG *et al*. 2003; Lynch SM *et al.* 1996;Nimse S, Pal D, 2015). Diketogulonic acid in turn is metabolized to xylose, xylonite, lyxonate, and oxalate with oxalate being a clinically significant end product of ascorbate metabolism(Cadenas E, Packer L, 2007). Molecular oxygen, with or without trace metals (iron, copper), superoxide, hydroxyl radicals, and hypochlorous acid, all can oxidize AA to DHA in biological systems (Cadenas E, Packer L, 2007; Colven RM, Pinnell SR, 1996).

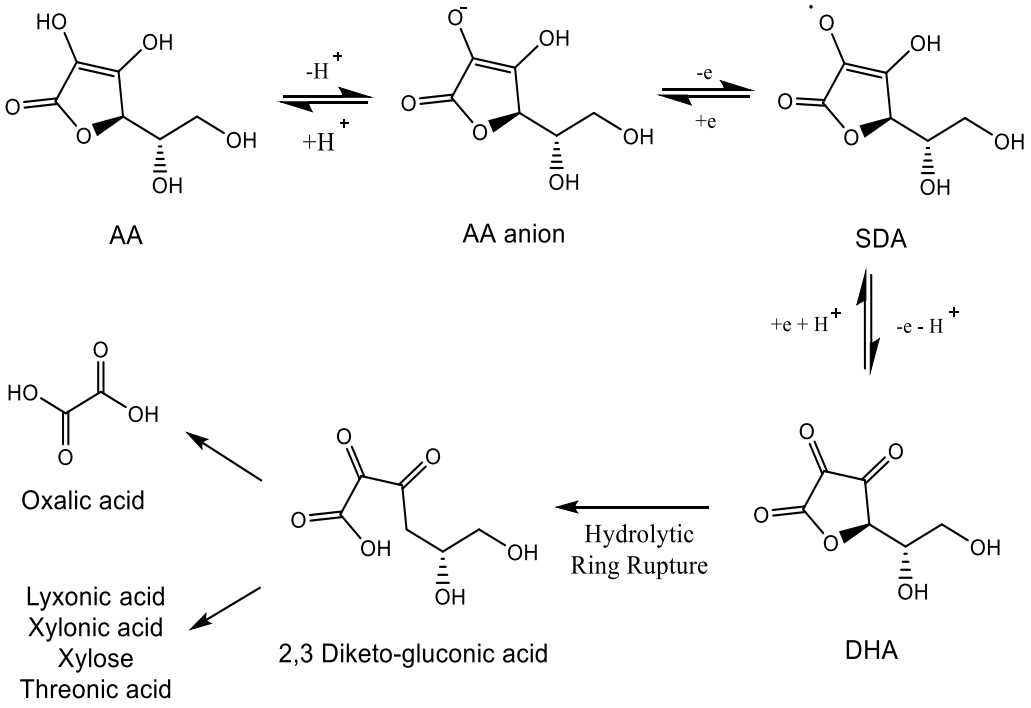


**Figure 2** Antioxidant mechanism of Ascorbic acid (AA) and its metabolism. AA protects organism due to oxidation of ascorbate to (semydehydroascorbic acid) SDA and then to DHA (dehydroascorbic acid) and has diverse functions to maintain the normal physiological state in humans. Adapted from Cadenas & Packer,2007.

## *Skin effects*

### *i. Ascorbic acid as skin antioxidant*

Ascorbic acid (AA), the most abundant antioxidant in human skin, forms a part of the complex group of enzymatic and non-enzymatic antioxidants that co-exist to protect the skin from reactive oxygen species (ROS) (Cadenas E, Packer L, 2007; Telang P, 2013). As AA is water soluble, it functions in the aqueous compartments of the cell, it becomes the major aqueous phase reductant (Pinnell SR, 2003; Santos M, 2014).

As previously described, AA has a reducing agent behaviour. Hence, AA donates two electrons sequentially, and when it loses these electrons becomes oxidized vitamin. Another substance is reduced. In this way, vitamin prevents oxidation of the reduced substance (Pinnell SR, 2003; Nimse S, Pal D, 2015).

Bendich, Machlin, Scandurra, Burton, and Wayner proposed the antioxidant mechanism of AA when it interacts with reactive specie (Oroian M *et al*. 2015). At physiological pH, the ascorbate anion is the predominant form present, due to the acidic nature of AA. This compound can undergo a reversible oxidation process and forms DHA, with ascorbyl radical formation. The ascorbyl radical is relatively unreactive and may react with other free radicals and the propagation of free radical reactions may be stopped (Pham-Huy L *et al*. 2008; Segal A, Moyano A, 2008; Pinnell SR, 2013; Stojiljkovic D *et al*. 2014; Pisoschi AM, 2015).

However, vitamin C is not able to act in lipophilic compartments (Olabisi 2004, Telang 2013). Therefore, AA cannot scavenge lipophilic radicals directly within the lipid compartments. In this case, this hydrophilic antioxidant acts as a synergist with tocopherol (TP) for the reduction of lipid peroxide radicals (Cadenas E, Packer L, 2007; Stamford NP, 2012; Pinnell SR 2013).

### *ii. Ascorbic acid in photoprotection and skin damage*

The topical application of AA increase cutaneous levels of this vitamin, and these is associated with protection of the skin from UVB-induced oxidative damage as measured by decrease in UVB erythema and sunburn cell formation (Burke KE, 2004; Stamford NP, 2012). The level of AA diminishes with exposure of UV light; nevertheless, its ability in neutralization the ROS (superoxide anion, peroxide and singlet oxygen) formed due to UV exposure was demonstrated, being equally effective against both UVB (290-320 nm) and UVA (320-400 nm) (Telang P, 2013). These ROS present harmful potential to start chain or cascade reactions that damage the cells, resulting in direct chemical alterations of the cellular DNA, the cell membrane and the cellular proteins, including collagen (Pinnell SR, 2003; Telang P, 2013).

Sunscreens when properly applied prevent UV-induced erythema and thymine dimer mutations that contribute to cutaneous carcinogenesis. Similar sunscreens preventing photoageing to decrease UV-induced erythema, sunburn cell formation and inducing collagen repair. Thus, UV protection can be optimized when combined filters with a topical antioxidant (Lopez-Torres M *et al.* 1988; Saral Y *et al*. 2002; Puvabanditsin P, Vongtongsri R, 2016; Almeida M *et al.* 2010). AA does not absorb UV light but in sunscreen exerts an UV-protective effect by neutralizing free radicals. As already suggested, AA alone can provide photoprotection, still works best in conjunction with TP, the vitamin E, which potentiates the photoprotection action of ascorbate against UV-induced erythema and diminished the number of sunburn cells as compared to that seen for each antioxidant individually which underlines the synergistic effects against UV-induced oxidative damage (Colven RM, Pinnell SR, 1996; Pinnell SR, 2003; Puvabanditsin P, Vongtongsri R, 2016; Stamford NP, 2012).

### *iii. Collagen synthesis*

AA is essential for collagen biosynthesis. It has been proposed that this vitamin influences quantitative collagen synthesis in addition to stimulating qualitative changes in the collagen molecule (Telang P, 2013). Once, ascorbate is evident in connective tissue and during collagen formation, acting as cofactor for the enzymes prolysyl and lysyl hydroxylase, both of which are required for the post-translational processing of types I and III collagen (Bisset DL, 2009; Masaki H, 2010, Stamford NP, 2012; Telang P, 2013). Additionally these enzymes are responsible for stabilizing and cross-linking the collagen molecules (Telang P, 2013; Santos M, 2014).

Another mechanism by which AA influences the collagen synthesis is by stimulation of lipid peroxidation, and the product of this process, malondialdehyde, in turn stimulates collagen gene expression (Bisset DL, 2009; Telang P, 2013).

AA also directly activates the transcription of collagen synthesis by increasing fibroblast proliferation resulting in greater collagen production and stabilizes procollagen mRNA (Stamford NP, 2012; Telang P, 2013). This role of AA in connective tissue is known at long time, but only since XVI century this evidence gained recognition, associated with signs and symptoms of scurvy (e.g. in blooding gums) (Telang P, 2013; Santos M, 2014). By these facts, AA might be presumed that possesses the potential to also increase collagen production for wrinkle appearance reduction (Stamford NP, 2012).

### *iii. Depigmenting agent*

AA interrupts the key steps of melanogenesis (Pinnell SR, 2003) resulting in a depigmentation action. AA is a depigmenting agent due to its inhibitory effect on tyrosinase (Masaki H, 2010). Vitamin C interacts with copper ions at the tyrosinase-active site and inhibits action of the enzyme tyrosinase, thus decreasing the melanin formation (Smaoui S *et al.* 2013; Telang P, 2013). So, vitamin C supresses melanin synthesis when entering skin cells and reduces significantly the production of melanin by inhibition tyrosinase activity. This results in a reduction of dopaquinone, an intermediate of melanin synthesis. Additionally, it converts exiting dopaquinone to L-dopa. Vitamin C can also reduce existing melanin exerting lightener of black melanin by a reduction reaction as schematized in Figure3 (Baertschi S, 2006; Hidrata L *et al*. 2014; Lorencini M *et al.* 2014; Olabisi A, 2004).

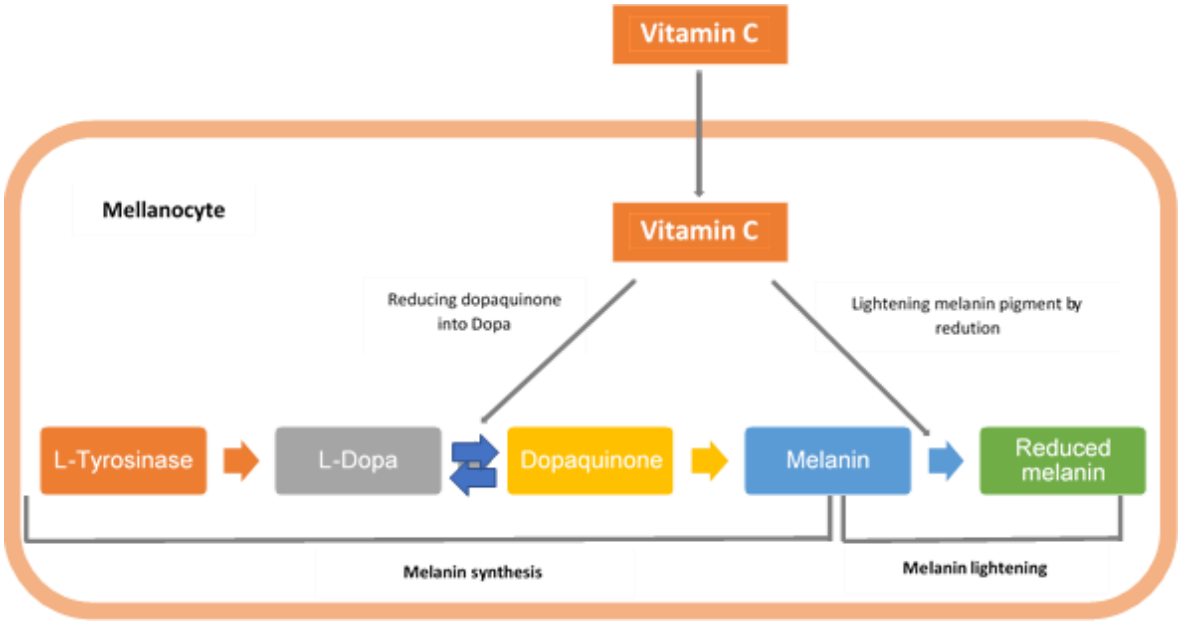


**Figure3** Skin lightning mechanism of vitamin C.

### *v. Anti-inflammatory action of vitamin C*

AA inhibits nuclear transcription factor kappa B (NFkB), which is responsible for the activation of a number of pro-inflammatory cytokines such as TNF-α, IL1, IL6 and IL8. Hence, AA has a potential anti-inflammatory activity and can be used in conditions like acne vulgaris and rosacea. Still, AA can promote wound healing and prevent post-inflammatory hyperpigmentation (Bisset DL, 2009; Stamford NP, 2012; Smaoui S *et al.* 2013; Telang P, 2013).

### *Topical pharmaceutical formulation aspects*

Orally, AA is absorbed at gut level but when given by supplementation in high doses since this process is limited by an active transport mechanism (Coronado MH *et al*. 2015; Lobo V *et al.* 2010; Lorencini M *et al.* 2014; Telang P, 2013). Accordingly, bioavailability of AA in the skin is inadequate when it is administered *per os*. So, topical application of this antioxidant is the only way to increase skin concentrations. Therefore, its use is favoured in the practice of dermatology (Colven RM, Pinnell SR, 1996, Haftek M *et al*. 2008, Sheraz M *et al*. 2011, Pinnell SR, 2003, Telang P, 2013). Conversely, due to inherent hydrophilicity, penetration of AA across the skin is poor (Stamford NP, 2012). To increase penetration of the epidermal barrier, aqueous formulations of AA must be at a pH below the pKa of AA itself (pKa 4.2), thus reducing the charge density on the molecule (Sheraz M *et al.* 2011). At pH lower than 3.5, the stability of AA is controlled and the ionic charge on the molecule is removed and its well transported across the *stratum corneum* (Stamford NP, 2012; Pinnell SR, 2013).

AA is available in the market as a variety of creams, serums and transdermal patches. Of these, only the serums contain active AA in an almost colourless form. It is unstable and, on exposure to light, gets oxidized to DHA, which imparts a yellow colour (Rowe *et al.* 2009, Stamford NP, 2012, Sui *et al.* 2014). Accordingly with its typical instability, the bulk material should be stored in an oxygen-impermeable non-metallic container, endangered from light, in a cool and dry place at low pH and minimum of water (Rowe, Sheskey *et al*. 2009, Stamford 2012, Sui *et al.* 2014).

Although AA is widely used in skin products to achieve clinical improvements, its poor skin penetration and its instability in formulations reduce its clinical efficacy as already described. To overcome these disadvantages, several AA derivatives, such as magnesium ascorbyl-2-phosphate (MAP), ascorbic acid 2-*O*-α-glucoside (AA2G), sodium ascorbyl phosphate (SAP), ascorbyl-6-palmitate (AP), and tetra-isopalmitoyl ascorbic acid, among others, have been synthetized and evaluated for their potential as pro-ascorbic acid derivatives (Austria R *et al*. 1997, Masaki H, 2010).

**Ascorbyl palmitate**

Ascorbyl palmitate (AP – Figure4), also known as vitamin C palmitate, L-ascorbyl-6-palmitate and 3-oxo-L-gulofuranolactone 6-palmitate is a synthetic ester comprised of 16-carbon chain saturated fatty acid palmitic acid and LAA. The ester linkage is at the carbon 6 of AA (Mauludin R *et al*. 2014). AP is prepared by condensing palmitoyl chloride and AA in the presence of a dehydrochlorinating agent such as pyridine (Godic A *et al*. 2014).

Therefore, AP is a fat-soluble synthetic ester of AA with lipophilic properties because of its chemical structure (Austria R *et al*. 1997; Meves A *et al*. 2012). Accordingly, the molecules of AP are orientated with the palmitic residue in the lipophilic phase and the cyclic ring in the aqueous phase (Segal A, Moyano A, 2008). Hydrolysis of ascorbyl palmitate yields AA and palmitic acid (Lupo MP, 2001).

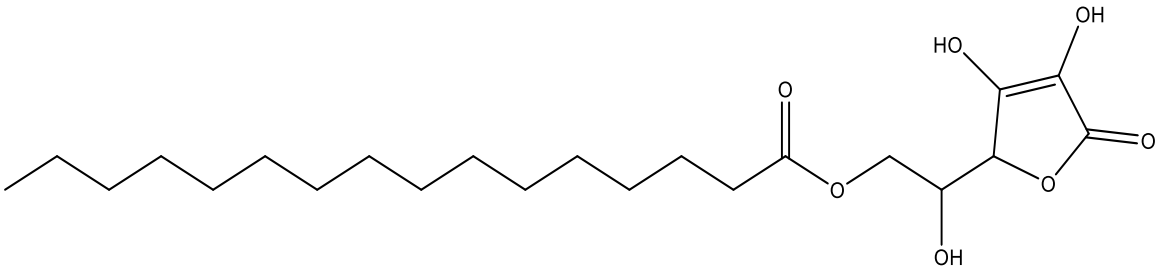


**Figure4** Ascorbyl palmitate structural formula.

The AP lipophilic character seems to be an advantage to penetrate the *stratum corneum* and also AP is pH neutral, making it non-irritating to the skin (Swern D, 1949; Segal A, Moyano A, 2008). In the following table – Table 2 – are summarized physical and chemical properties of this AA derivative (Mauludin R *et al*. 2014; Segal A, Moyano A, 2008; Smart RC, Crawford CL, 1991).

**Table 2** Physical and chemical properties of ascorbyl palmitate (Swern 1949, Rowe, Sheskey *et al*. 2009).

| Property | | Ascorbyl palmitate specifications |
|---|---|---|
| IUPAC name | | [2-(3,4-dihydroxy-5-oxo-2*H*-furan-2-yl)-2-hydroxyethyl] hexadecanoate |
| Appearance | | Odourless, white to yellowish powder |
| Formula | | $C_{22}H_{38}O_7$ |
| Molecular mass | | 414.54 |
| Density | | 1.15 g/cm$^3$ |
| pH | | Neutral pH (non-acidic) molecule |
| pKa | | 4.45 |
| Melting Point | | 107$^o$C-117$^o$ C |
| Solubility (g/ml) | Water | Practically insoluble: 0.0062 mg/ml |
| | Glycerol (USP) | 0.01 mg/ml |

Meves *et al*. 2002, was investigated the antioxidant effects of AP at cellular membranes. The antioxidant effect of AP has been recognized, because in its presence intracellular levels of ROS decreased. However, the same authors verified that lipid part of AP promotes UVB induced lipid peroxidation and formation of potentially toxic oxidized lipids that induces substantial cellular damage (Smart RC, Crawford CL, 1991; Perricone N, 1993; Meves A *et al*. 2012).

Other authors was found that AP is thirtyfold more effective than AA after topical application as an inhibitor of some of the biochemical parameters associated with tumour production in mice skin (Smart RC, Crawford CL, 1991). Likewise, AP when applied after UV burning reduces redness rather than patient that were untreated. The suspected mechanism is that AP acts as both an antioxidant and anti-inflammatory agent (Smart RC, Crawford CL, 1991; Perricone N, 1993; Lupo MP, 2001)

In terms of technological aspects, the cyclic ring of AP structure is sensitive to oxidation and can influence the formulation. In oil in water (O/W) emulsions, the cyclic ring (hydrophilic part of the molecule) is in the external aqueous phase, and with this, the stability of AP can be compromised (Segal A, Moyano A, 2008).

Other technological aspect is the fact that the combination of AP with α-tocopherol (TP) shows marked synergism, which increases the effect of the components and allows the amount, used to be reduced (Rowe *et al.* 2009). The solubility of AP in alcohol permits it to be used in non-aqueous and aqueous systems and emulsions.

Ascorbyl palmitate is touted as being good antioxidant to be incorporated in emulsions, creams, lotions, and oils (Lupo MP, 2001; Rowe *et al*. 2009; Pinnell SR, 2013).

Moreover, AP due to its amphiphilic character is able to penetrate the skin better and was shown to have better stability than AA (Smart RC, Crawford CL, 1991; Perricone N, 1993; Meves A *et al*. 2012; Mauludin R *et al.* 2014). Furthermore, AP appears to remain on the extracellular surface of cells and may not be readily converted to LAA (Smart RC, Crawford CL, 1991; Perricone N, 1993; Meves A *et al*. 2012; Mauludin R *et al.* 2014).

The main problem of AP is, its oxidation mediated by transition metal ions presented in traces. Besides oxygen, light can also accelerate oxidative degradation of AP (Meves A *et al*. 2012). AP is stable in the dry state, but is gradually oxidized and becomes discoloured when expose to light and high humidity. In an unopened container, stored in a cool place, it has a shelf life of at least 12 months (Meves A *et al*. 2012; Mauludin R *et al.* 2014).

**Ascorbic acid-2-glucoside**

Ascorbic acid-2-*O*-glucoside (AA2G– Figure5), is a glycoside of disaccharide in which glucose and AA are bound with an 1,2-linkage (Nakamura S, Oku T, 2009).

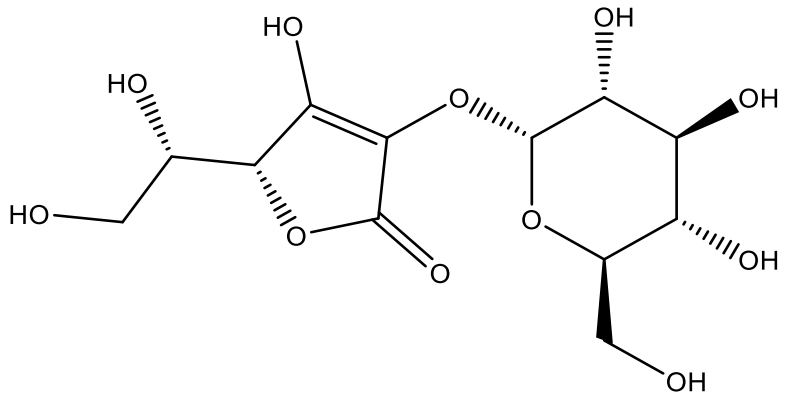


**Figure5** Ascorbic acid-2-glucoside structural formula.

Ascorbic acid-2-glucoside or 2-*O*-α-D-glucopyranosyl-L-ascorbic acid is a stable derivative of AA which is efficiently synthesized by regioselective transglucosylation with α-glucosidase and cyclodextrin glucosyltransferase (CGTase) (Nakamura S, Oku T, 2009; Nakazawa H *et al.* 2012; Nayama S *et al*. 1999). Glycosylation of ascorbate at the 2-O position improves the stability and its feasibility as a radical scavenger has been reported (Nakamura S, Oku T, 2009). The hydroxyl group of the second carbon in AA is highly reactive and plays an important role in the biologic activities. However, it can also be the site of inactivation and decomposition. AA2G is produced from AA and starch by use of enzymes that reversibly bind glucose to the reactive hydroxyl group to protect the site from destruction (Nakamura S, Oku T, 2009).

Table 3 summarizes the main physical and chemical properties of ascorbic acid-2-glucoside.

**Table 3** Physical and chemical properties of ascorbic acid-2-glucoside (Takeuchi 2006, 2016).

| Property | | Ascorbic acid-2-glucoside specifications |
|---|---|---|
| IUPAC name | | (2*R*)-2-[(1*S*)-1,2-dihydroxyethyl]-3-hydroxy-4-[(2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2*H*-furan-5-one |
| Appearance | | White to yellowish white powder, or crystalline powder |
| Formula | | $C_{12}H_{18}O_{11}$ |
| Molecular mass | | 338.265 |
| Density | | 1.83g/cm$^3$ |
| pH | | 2.3-2.4 |
| pKa | | N/A |
| Melting Point | | N/A |
| Solubility (g/ml) | Water | 1.25 |
| | Glycerol (USP) | N/A |

N/A – Information not available

*In vivo*, AA2G is easily hydrolysed by α-glucosidase, an enzyme present in the membrane of skin cells, (Tagawa M, Tabata Y, 1988; Takebayashi J *et al*. 2002; Stamford NP, 2012) exhibiting biological activities as an antiscorbutic effect, collagen synthesis-enhancing effect melanin synthesis-inhibiting effect, antioxidative as free radical scavenging activities also with metal chelating activity and reducing ability and anti-aging effects (Huang WY *et al*. 2013; Nakamura S, Oku T, 2009). AA2G is hydrolysed over a prolonged period, resulting in consistent and sustained beneficial physiological effect on the skin (Tai A *et al*. 2014; Tai A *et al*. 2003; Takebayashi J *et al.* 2008; Takebayashi J *et al.* 2006; Takebayashi J *et al*. 2002).

It has showed that even though AA2G has a higher stability its antioxidative capacity was lower than the activity *in vivo* of AA and AP. This can be explained by poor penetration of this compound through human *stratum corneum* due to its high hydrophilicity (Tai A *et al*. 2003; Takebayashi J *et al*. 2008).

AA2G has no cytotoxicity becoming an excellent AA derivative for topical application (Tai A *et al*. 2003; Takebayashi J *et al*. 2008). Accordingly, AA2G has been widely

used in cosmetic products practically used as a skin lightening cosmetic ingredient (Tai A *et al*. 2003; Takebayashi J *et al*. 2008). AA2G has superior formulation stability being able to resist discolouration and degradation, while retaining all biologic activities that provide lightening, UV damage protection and anti-aging properties (Tai A *et al*. 2003; Takebayashi J *et al*. 2008). AA2G is highly stable in solution even at high temperatures, low pH and in presence of metal ions. These facts help maintaining the final product quality (Takebayashi J *et al.* 2008; Takebayashi J *et al.* 2006; Takebayashi J *et al*. 2002). However, the degradation of this derivative is triggered by the temperature change and pH variation. The stability of this antioxidant as previously described is influenced by pH. For this reason, leaving it under prolonged conditions of strong acidity or alkalinity (pH 2-4 or pH 9-12) should be avoided (Takebayashi J *et al.* 2008; Takebayashi J *et al.* 2006; Takebayashi J *et al*. 2002). These variations were very critical for determining its bio functionality of AA2G in skin. The optimal condition of retaining AA2G with the highest stability was determined to be 55.3ºC and pH 6.4 (Takebayashi J *et al.* 2006; Takebayashi J *et al*. 2002).

**Magnesium ascorbyl phosphate**

Magnesium-L-ascorbyl-2-phosphate (MAP – Figure 6) is a stable water-soluble ascorbyl ester as inorganic precursor of vitamin C that ensures delivery of vitamin C to the skin (Austria R *et al*. 1997; Smaoui S *et al.* 2013; Telang P, 2013) exhibiting antioxidative effects (Tagawa M, Tabata Y, 1988).

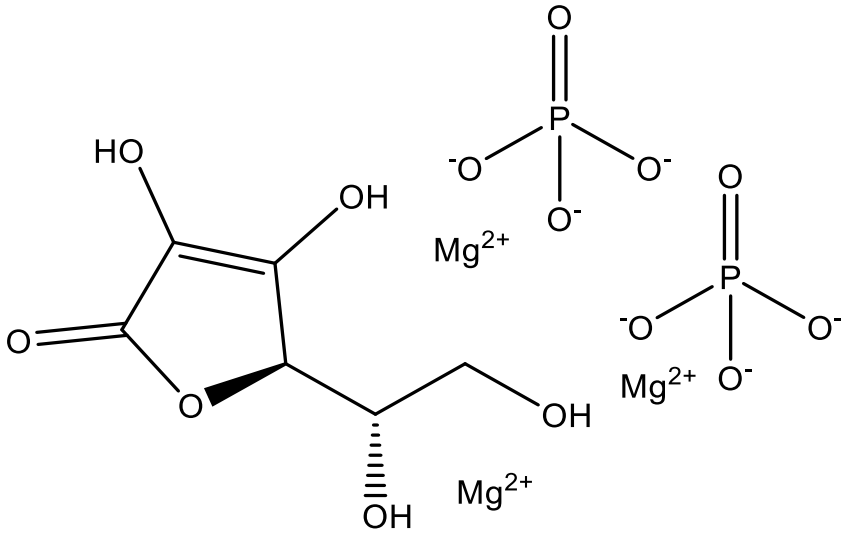


**Figure 6** Magnesium ascorbyl phosphate structural formula.

MAP is obtained by the esterification reaction of a hydroxyl group of AA with an inorganic chain, which protects the enediol ring from the degradation reactions (Tagawa M, Tabata Y, 1988). The hydroxyl group is modified by an inorganic phosphoric ester group introduced in position 2.

In Table 4 are shows physical and chemical properties of magnesium ascorbyl phosphate.

**Table 4** Physical and chemical properties of magnesium ascorbyl phosphate (Silva and Campos 2000, Smaoui 2013).

| Property | | Magnesium ascorbyl phosphate specifications |
|---|---|---|
| IUPAC name | | trimagnesium (2*R*)-2-[(1*S*)-1,2-dihydroxyethyl]-3,4-dihydroxy-2*H*-furan-5-one; diphosphate |
| Appearance | | White or yellowish powder |
| Formula | | $C_6H_8Mg_3O_{14}P_2$ |
| Molecular mass | | 759.22 |
| Density | | N/A |
| pH | | 7 |
| pKa | | N/A |
| Melting Point | | >300ºC |
| Solubility (g/ml) | Water | 1.54 |
| | Glycerol (USP) | N/A |

N/A – Information not available

MAP presents instability in presence of metal ions. This antioxidant is unstable under UV light, and is stable at 50ºC at least for 10 weeks (in aqueous solution) and 80ºC at least for 20 hours (Austria R *et al*. 1997, Stamford NP, 2012). When compared with AA and AP, MAP showed better stability after 60 days confirming that the phosphoric group has the capability of protect the enediol system from hydrolysis (Kameyama K *et al.* 1996; Silva GM, Maia Campos PM, 2000; Smaoui S *et al.* 2013; Tagawa M, Tabata Y, 1988). Unlike AA and AP that are sensitive to high pH and have a better stability at pH 3, MAP is stable at pH 7, like SAP. One study showed that this compound is highly unstable at acid pH (Austria R *et al*. 1997; Stamford NP, 2012).

MAP also demonstrates an inhibitory effect on melanogenesis, suppressing melanin formation by tyrosinase activity and melanoma cells and, thus, decrease the skin pigmentation, resulting in spots removal and whitening effect (Tai A *et al.* 2014; Tagawa M, Tabata Y, 1988). Thus, MAP has been mainly utilized as an active ingredient of whitening cosmetics (Kameyama K *et al.* 1996; Silva GM, Maia Campos PM, 2000; Smaoui S *et al.* 2013; Tagawa M, Tabata Y, 1988).

MAP exhibited *in vitro* evidence that crossing the epidermis is converted to AA. In an *in vitro* study of monolayer human fibroblast cultures, this derivative was found to be equivalent to AA in stimulating collagen synthesis and was stable at a neutral pH (Kameyama K *et al.* 1996; Silva GM, Maia Campos PM, 2000; Smaoui S *et al.* 2013; Tagawa M, Tabata Y, 1988). Other *in vitro* studies in human skin fibroblast in culture demonstrated enhanced collagen synthesis and cell growth (Lupo MP 2001; Smaoui S *et al.* 2013).

Recent investigations showed various possibilities to utilize MAP as multi-functioning, stable 'provitamin C'. Intradermal and extradermal protective effects against UV-generated radicals suggest its use for UV-care products. The results imply that MAP can protect the keratinocytes against UVA irradiation, possibly via increasing the levels of GSH (Kameyama K *et al.* 1996; Silva GM, Maia Campos PM, 2000; Smaoui S *et al.* 2013; Tagawa M, Tabata Y, 1988).

As clinical studies strongly support that acne, can be considered an oxidative stress-mediated disease, it is another condition where MAP could be used. Lee, W. *et al*., in 2016 showed that MAP has mild anti-inflammatory and antioxidative effects in cultured sebocytes (Kameyama K *et al.* 1996; Hong J *et al*. 2004). Thus, this derivative was considered a complementary therapy for the regulation of inflammatory processes (e.g. in acne). An *in vitro* study showed that this derivative can be used in acne vulgaris, reducing the inflammatory acne and preventing sebum oxidation (Kameyama K *et al.* 1996; Silva GM, Maia Campos PM, 2000). This same study verified a decrease in lipid peroxidation after treatment with MAP (Puvabanditsin P, Vongtongsri R, 2016; Stamford NP, 2012; Smaoui S *et al.* 2013).

This derivative has greater stability than AA when added in formulations with high aqueous content and neutral or slightly alkaline pH. According to an *in vitro* study of the cutaneous penetration of AA and MAP, both active ingredients showed skin penetration capacity from cosmetic formulations (Silva GM, Maia Campos PM, 2000). The same authors demonstrated that MAP increases intra and extracellular hydration. MAP when penetrating the epidermis releases free vitamin C, by the action of the phosphatases enzymes (Tagawa M, Tabata Y, 1988; Lupo MP, 2001). In epidermis, exerts similar functions to AA.

**Sodium ascorbyl phosphate**

Sodium ascorbyl-2-phosphate (SAP – Figure 7) is water-soluble consisting of a stabilized sodium salt of L-ascorbic acid. AA suffers esterification at position 2 which confers protection from oxidation (Spiclin P *et al.* 2013, Klock J *et al.* 2005). The introduction of the phosphate group in position 2 of the cyclic ring protects the enediol system against oxidation (Kanatate T *et al.* 2005; Spiclin P *et al.* 2013; Klock J *et al.* 2005). This also means that unlike AA, this compound cannot be used as an antioxidant agent to stabilize formulations. This hydrophilicity also decreases the molecule's ability to penetrate the skin. The AA molar activity of the phosphorylated ester of vitamin C is equivalent to LAA itself (S piclin P *et al.* 2013, Prospector 2016).

SAP is a stable AA derivative, which is cleaved *in vivo* by enzymes in the skin to release and ensure a constant delivery of vitamin C into the skin (Klock J *et al.* 2005; Aparajita V, Ravikumar P, 2014).

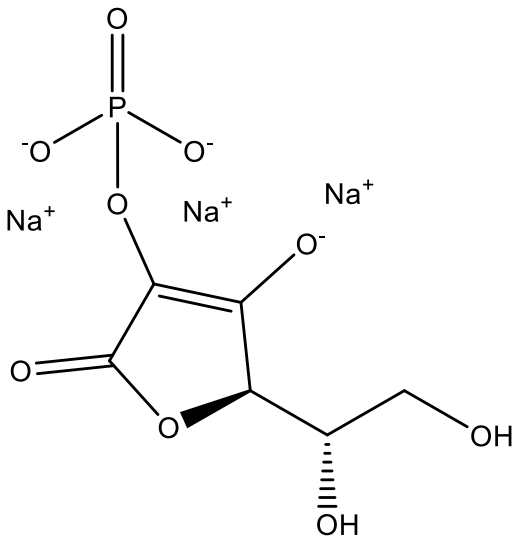


**Figure 7** Sodium ascorbyl phosphate strutural formula.

In Table 5 the main physical and chemical characteristics of sodium ascorbyl phosphate are summarized.

**Table 5** Physical and chemical properties of sodium ascorbyl phosphate (Nayama S 1999, Spiclin P 2003).

| Property | | Sodium ascorbyl phosphate specifications |
|---|---|---|
| IUPAC name | | trisodium (2*R*)-2-[(1*S*)-1,2-dihydroxyethyl]-3,4-dihydroxy-2*H*-furan-5-one; diphosphate |
| Appearance | | White to pale beige powder with practically no odour |
| Formula | | $C_6H_60_9Na_3P$ |
| Molecular mass | | 322.05 |
| Density | | N/A |
| pH | | 9-10 |
| PKa | | N/A |
| Melting Point | | N/A |
| Solubility (g/ml) | Water | 7.89 |
| | Glycerol (USP) | N/A |

N/A – Information not available

SAP is a less reactive precursor of vitamin C. Being a prodrug it must be first cleaved by enzymes (presumably an alkaline phosphatase) in the skin to release free AA before achieving the target tissue and protecting the cells against free radicals (Austria R *et al*. 1997; Shalmashi A, Eliassi A, 2008; Smaoui S *et al.* 2013).

The SAP is one of the most effective free radical quenchers. It protects the cells against free radicals, promotes collagen formation and acts on the melanin formation process (Segal A, Moyano A, 2008; Smaoui S *et al.* 2013). The addition of this bioconvertible antioxidant improves photoprotection by being converted to vitamin C within the skin. The bioconversion forms an antioxidant reservoir that deactivates the ROS generated by the UV photons that the sunscreens do not block in the *stratum corneum* (Aparajita V, Ravikumar P, 2014; Kanatate T *et al.* 2005; Sui X et al. 2014). SAP acts on melanin formation process in the skin preventing hyperpigmentation, and therefore can be used in skin whitening preparations (Olabisi A, 2004; Segal A, Moyano A, 2008; Smaoui S *et al.* 2013; Stamford NP, 2012; Park KT *et al*. 2011). There is an evidence showing that SAP is highly stable at 43°C at pH 7 or higher (Aparajita V, Ravikumar P, 2014).

### 3-*O*-Ethylascorbic acid

FIGURE 9

3-*O*-Ethyl-L-ascorbic acid (3OAA –Figure 8), is an ascorbic acid derivative with a simple structure and efficient transdermal activity used in commercial cosmetics (Tai A *et al*. 2014; Tai A *et al*. 2003).

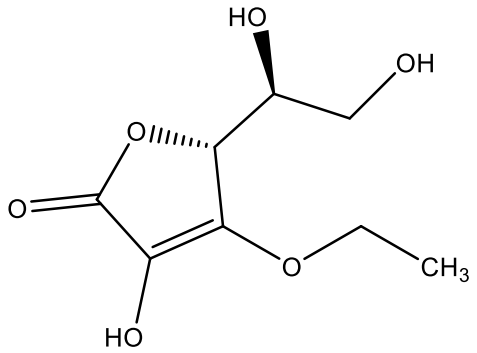


**Figure 8** 3-*O*-Ethylascorbic acid structural formula.

The 3OAA derivative is an example of an approach that intent making AA more lipophilic. To obtain this derivative, alkylation only at the 3-OH group is necessary (Tai A *et al.* 2003; Jin S, Miao X, 2008). The 3-*O*-ethyl derivative masks the 3-OH, preventing its ionization and the subsequent oxidation of vitamin C. The 3-*O*-ethyl derivative is also more lipophilic than AA because of the added lipophilic ethyl group and hence exhibit improved permeation into the skin (Jin S, Miao X, 2008; Nikki T *et al.* 2016).

In Table 6 the main physical and chemical characteristics of 3-*O*-ethyl ascorbic acid are summarized.

**Table 6** Physical and chemical properties of 3-*O*-ethylascorbic acid.

| Property | | 3-*O*-Ethylascorbic acid specifications |
|---|---|---|
| IUPAC name | | (2*R*)-2-[(1*S*)-1,2-dihydroxyethyl]-3-ethoxy-4-hydroxy-2*H*-furan-5-one |
| Appearance | | white, odourless, crystallized powder |
| Formula | | $C_8H_{12}O_6$ |
| Molecular mass | | 294.18 |
| Density | | N/A |
| pH | | 3-4.5 |
| pKa | | N/A |
| Melting Point | | 111-116ºC |
| Solubility (g/ml) | Water | N/A |
| | Glycerol (USP) | N/A |

N/A – Information not available

3OAA acts as a radical scavenging, boosts collagen synthesis, balances skin tone and prevent photo-ageing. Also, 3OAA helps in DNA protection, sun protection and has anti-inflammatory effects (Tai A, 2003; Stamford NP, 2012, Tai A, 2014). 3OAA compound can inhibit the activity of tyrosinase, thus inhibiting the synthesis of melanin by acting on $Cu^{2+}$. In addition, 3OAA can also block the polymerization of dihydroxyindole, which is an intermediate in melanogenesis (Olabisi A, 2004; Segal A, Moyano A, 2008; Stamford NP, 2012; Nikki T *et al.* 2016).

Even though 3OAA has positive skin whitening effects, its high-water solubility unfortunately indicates that it is likely AA difficult to permeate across the *stratum corneum* and to be delivered into the epidermis. Therefore, it is supposed to possess good compatibility for hydrophilic drugs owing to the polarity part of the amphiphilic molecules (Segal A, Moyano A, 2008; Stamford NP, 2012; Smaoui S, 2013).

3OAA is very stable at different temperatures, pH and in the presence of oxygen and light. Similarly, to other derivatives, it has an excellent whitening effect, high antioxidant capacity, acts on inflammation, enhances the elasticity of the skin and repairs skin cells by accelerating the synthesis of collagen. However, of these characteristics, to our current knowledge only the whitening effect was tested *in vivo* (Tai A *et al.* 2003; Tai A *et al.* 2014, Nikki T *et al.* 2016).

**The new ascorbic acid derivative: sulphate ascorbic acid-2-glucoside**

A new sulphate derivative of ascorbic acid-2-glucoside was synthetized in the Laboratory of Organic and Pharmaceutical Chemistry at Faculty of Pharmacy, University of Porto (unpublished results). The new compound sulphate ascorbic acid-2-glucoside (AA2GS) is shielded by the introduction of sulphates groups in ascorbic acid-2-glucoside (Figure 9).

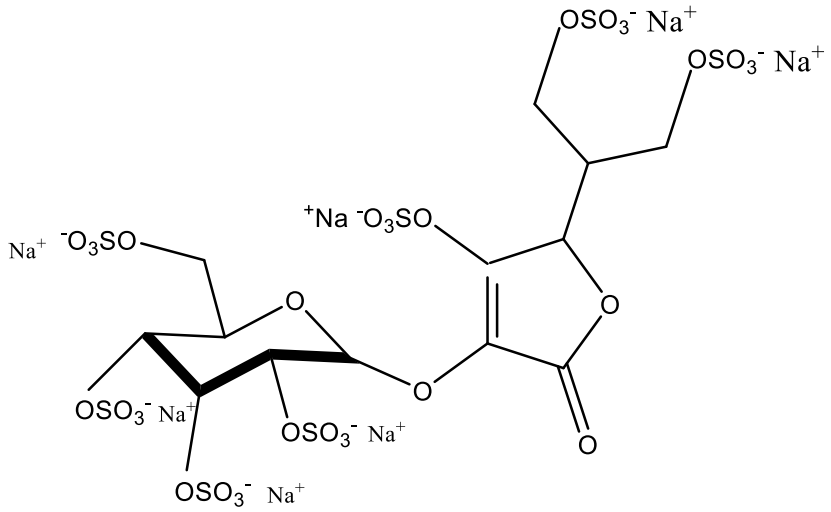


**Figure 9** Sulphate ascorbic acid-2-glucoside structural formula.

In Table 7 chemical data about this new compound are presented.

**Table 7** Physical and chemical properties of sulphate ascorbic acid-2-glucoside.

| Property | Sulphate ascorbic acid-2-glucoside specifications |
|---|---|
| Appearance | white, odourless, crystallized powder |
| Formula | $C_{13}H_{13}Na_7O_{31}S_7$ |
| Molecular mass | 1050 |

# Chapter II: Aims

## Charpter II: Aims

Ascorbic acid (AA) has important physiological effects on skin, including inhibition of melanogenesis, promotion of collagen biosynthesis and prevention of free radical formation, all closely related to the well-known antioxidant properties of this compound (Colven RM, Pinnell SR, 1996; Austria R *et al.* 1997; Lupo MP, 2001; Meves A *et al.* 2002; Segal A, Moyano A, 2008; Telang P, 2013). Vitamin C therefore plays an important role in skin aging and may be considered an interesting ingredient of cosmetic skin care products (Colven RM, Pinnell SR, 1996, Hacisekvi A, 2009; Haftek M *et al*. 2008; Hanada Y *et al*., 2014; Hidrata L *et al*. 2014; Hong J *et al*. 2004; Hovorka S, Schöneich C, 2001; Humbert PG *et al.* 2003; Hurtado N *et al*. 2009; Segal A, Moyano A, 2008; Telang P, 2013).

Antioxidants, due to their action in prevention of skin aging have been increasingly used in cosmetic products. In development of new high quality anti-aging cosmetic preparations, pre-formulation studies and validation of analytical methods are required (Silva GM, Maia Campos PM, 2000; Almeida M *et al*. 2010; Bharate S *et al*. 2011; Bezerra G *et al*. 2016). AA easily losses its biologic activity because of heat, oxidation, and reactions with metal ions and other common cosmetic ingredients. These reactions occur quickly in basic conditions and the compound degrades itself irreversibly in a biologically inactive form (2,3-diketo-L-gulonic acid). This causes discolouration in the cosmetic formulations and dramatically decreases the ability of AA to provide the benefits of younger looking healthier skin. To overcome these problems, chemist professionals developed new substances consisting in vitamin C derivatives (Lupo MP, 2001; Puvabanditsin P, Vongtongsri R, 2016; Telang P, 2013; Santos M, 2014) where AA is chemically modified by esterification of the hydroxyl group with long-chain organic or inorganic acids (Austria R *et al.* 1997; Stamford NP, 2012; Sheraz M *et al*. 2011; Smaoui S *et al.* 2013).

Although several derivatives were introduced along the years, to our knowledge a comprehensive comparison study has not been yet performed. Therefore, the main objective of this dissertation was to compare six derivatives of AA: ascorbyl palmitate (AP), magnesium ascorbyl phosphate (MAP), sodium ascorbyl phosphate (SAP), 3-O-ethylascorbic acid (3OAA), ascorbic acid-2-glucoside (AA2G) and sulphate ascorbic acid-2-glucoside (AA2GS) in respect to their solubility, stability, and antioxidant activity.

The selection of these antioxidants is based on a previous study that investigated anti-aging cosmetics. The work aimed to appreciate how anti-aging formulations changed in

the past five years regarding 25 pure antioxidants composition. Data were collected from anti-aging formulations commercialized in 27 main stores and pharmacies in the Portuguese market. The study started on 2011 and was updated 28 with products launched or whose composition has been renewed after 2013 and 2015. Overall, this study 32 pointed out that ascorbic acid and tocopherol and their derivatives are the most used antioxidants 33 in anti-aging cosmetics commercialized on the Portuguese market (Silva S, 2016). The derivatives of AA involved in these dissertations were the most popular between among of antioxidant active ingredients in anti-aging cosmetics. A new ascorbic acid derivative (AA2GS) was added in this comparative study.

The specific aims were:

i) To determine in same conditions the solubility of each compound in water and glycerol by flask method.

ii) To establish the stability of ascorbic acid and its derivatives. It was examined its behaviour versus time in some special conditions that accelerate the degradation of compounds. Samples were periodically analysed by spectrophotometric method (UV/Vis):

a. pH stability

b. thermal stability

c. stability in the presence of metals ($CaOH_2$, $CuSO_4$, $FeCl_3$, $FeSO_4$, $MgSO_4$).

iii) To study the photostability of the compounds.

iv) To define their compatibility with widely used excipients used in topical and cosmetic formulations by differential scanning calorimetry technique (DSC).

v) To evaluate the antioxidant activity of all compounds assessed by DPPH assay.

Ultimately, this study would provide the information of which compound is more stable in every situation knowing that stability is a core quality attribute for any viable active ingredient product (Sheraz M *et al*. 2011; Obonga W *et al.* 2013; Mauludin R *et al*. 2014).

# Chapter III: Materials and Methods

# Chapter III: Materials and Methods

## III.1 General reagents

*Antioxidants:*

Ascorbic acid was obtained from Sigma-Aldrich (Lot: 05316HJ-508 Cat: A9, 290-2), sodium ascorbyl phosphate (STAY-C® 50) from DSM (Lot: UE01603035), ascorbic acid 2-glucoside from DKSH (Lot: DGF), ascorbyl palmitate from DSM Lot: UQ60306047), magnesium ascorbyl phosphate from DSM (Lot: 0418595007), 3-O-ethylascorbic acid from Selco (Lot: 72461404). The sulphate ascorbic acid-2-glucoside was synthesized in LQOF.

*Others:*

Glycerol was obtained from Acofarm (Lot: 15-18-17-P-1), potassium chloride was obtained from Merck (Lot: 1049360500); hydrochloric acid from Fischer Chemical (Lot: 1546962); citric acid monohydrate from Aldrich (Lot: 28252-099); sodium hydroxide from VWR Chemicals (Lot: 14J270024); trisodium citrate dehydrate from Merck (Lot: 1064480500); disodium hydrogen phosphate from VWR ProLabo (Lot: 12G18007) and phosphoric acid from Panreac (Lot: 0000184231), iron (III) chloride was obtained from Sigma-Aldrich (Lot: 04535IJ209); iron(II) sulfate heptahydrate from Merck (Lot: 045284950); copper(II) sulphate pentahydrate from Sigma-Aldrich (Lot: 023435CD609); magnesium sulphate monohydrate from Sigma-Aldrich (Lot: 02843MS304); calcium hydroxide from Cromoline (Lot: 44242/12), carbopol 980 from Noveon (Lot: EC6D3CC072); Miglyol 812 from Acofarm (Lot: 132249); EDTA from Sigma Aldrich (Lot: 059K0022), tocopherol from Sigma Aldrich (Lot: 1913342), potassium cetyl phosphate from AAKO (lot: C12070802); stearyl alcohol from Acofarm (lot: 150896-P-1), hydroxyethyl cellulose from Fragon (Lot: L13030090-OF-148212), rutin hydrate was obtained from Sigma (Lot: 073K0099), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was purchased from Sigma–Aldrich (USA).

### III.2 General equipment

- A Jasco V-650 UV/Visible spectrophotometer dual beam (Jasco, Japan) was used equipped with deuterium UV lamp and Vis QI interfaced with Windows program of computer via Spectra ManagerTM II software data system program. Two open-top UV quartz cell with 1 cm length at wavelength 280 nm were used.

- Ultra sonic devise (ultra sonicator) to help dissolving antioxidants in solution samples (Selecta, Spain).

- A pH meter to measure the pH of each buffer solution (Jenway 3510, United Kingdom).

- A light source: fluorescent lamp that was designed to produce an artificial daylight combining Vis and UV outputs and a radiometer Accumeter UVM-7 radiometer (Accumeter, Germany).

- A DSC 200F3 Maia (Netzsh–Gerätebau GmbH) instrument.

### *III.3. Solubility in water and glycerol*

To analyse the solubility of AA, AP, MAP, SAP, 3OAA, AA2G in deionized water an excess of solid sample was added to the medium in a flask, and the resulting suspension was shaken until equilibrium solubility (i.e., thermodynamic solubility) was reached, at 25ºC after 2 h. After this time, undissolved sample was then removed by filtration, and the concentration of the compound in the filtrate was determined (Avdeef A, 2001; Henchoz Y *et al*. 2009). The solubility was defined according to United States Pharmacopeia (USP) (USP 2015) and Portuguese Pharmacopeia (F.P.(VIII) 2008).

Briefly, three 5 ml test tubes for each substance in study were filled with water as solvent and an excess. The test tubes were submerged in water bath at 40 ºC by 10 minutes. Then, the samples were fixed in sonication device for 15 minutes. After this, the solutions are cooled to room temperature (25ºC) and reached the equilibrium in absence of the light through 2 h. After this, the solutions were filtrated through membranes 25 mm syringe filters – 0.45µm polypropylene membrane, VWR® to exclude all remaining solids particles. The blank (water) was submitted the same treatment to eliminate possible interferences.

Five independent standard solutions were prepared under the same conditions of saturated concentrations at 0.165, 0.250, 0.330, 0.450 and 0.500 mg/mL. Hence, exactly known masses of each antioxidant was diluted in precise volume of solvent to obtain a concentration range that was measured in spectrophotometer at the same wavelength (λ=280nm).

Subsequently dilutions of the filtrates were scanned in the range of 200-800 nm to obtain the spectrum of each antioxidant. Posteriorly, these data allowed to select a common and appropriate wavelength for determination of solubility of each analyte.

After filtration, 1 ml of each solution of antioxidant was analysed in spectrophotometric method and interpolated into the several calibrations curves. Solutions concentrations were determined from the means obtained in the different calibration curves.

Glycerol solubility measurement at 25ºC under equilibrium of 2h was performed by a similar procedure utilized for water solubility.

### *III.4. Stability*

#### *III.4.1. pH*

Buffer solutions with pH values 2, 3, 4, 5, 6, 7 and 8 were prepared and the pH of each buffer solution was measured with a pH meter. The buffer solutions of pH 2, 3, 4, 5, 6, 7 and 8 were prepared according to Portuguese Pharmacopeia 8.0 specifications (F.P.(VIII) 2008) and the others by Protocols and Application Guide (Guide 2004). Thus, the composition of each buffer solution was:

*Buffer solution pH 2.0* 6.57 g of *potassium chloride* was dissolved in *water* and added 119.0 ml of *0.1 M hydrochloric acid*. Dilute to 1000.0 ml with *water*.

*Buffer solution pH 3.0* 21.0 g of *citric acid* was dissolved in 200 ml of 1 M *sodium hydroxide* and diluted to 1000 ml with *water* R. After this, 40.3 ml of this solution was diluted to 100.0 ml with 0.1 M *hydrochloric acid*.

*Buffer solution pH 4.0* a mixture of 59.0 ml of 0.1 M citric acid *monohydrate* with 41 ml of 0.1 M of *trisodium citrate dehydrate* was made.

*Buffer solution pH 5.0* a solution containing 20.1 g/l of *citric acid* and 8.0 g/l of *sodium hydroxide* was prepared. Adjust the pH with dilute *hydrochloric acid*.

*Buffer solution pH 6.0* 63.2 ml of a 71.5 g/l solution of *disodium hydrogen phosphate* and 36.8 ml of a 21 g/l solution of *citric acid* were mixed.

*Buffer solution pH 7.0* 82.4 ml of a 71.5 g/l solution of *disodium hydrogen phosphate* with 17.6 ml of a 21 g/l solution of *citric acid* were mixed.

*Buffer solution pH 8.0* 20 g of *dipotassium hydrogen phosphate* was *d*issolved in 900 ml of *water.* Adjust the pH with *phosphoric acid R*. Dilute to 1000 ml with *water.*

A 100 µM of antioxidant solution buffered at pH 2, 3, 4, 5, 6, 7 and 8 was prepared in triplicate. These solutions were reserved at room temperature (20ºC) in dark space. Aliquots of 3.00 ml were transferred to cuvettes at predetermined time intervals over 0, 1 hour, 2 hours, 1 day, 8 days and 21 days and their absorbance measured by UV-Vis spectrophotometry. The blank of each pH corresponded to 3.00 ml of respective buffer.

### *III.4.2. Temperature*

A 100 µM of each antioxidant solution buffered at pH 5 was prepared in triplicate. Other three samples of these solutions were reserved at 4ºC, 40ºC, 25ºC and 60ºC in dark space with absence of the light.

Aliquots of 3.00 ml were transferred to cuvettes at predetermined time intervals over 0, 1 hour, 2 hours, 1 day, 8 days and 21 days and their absorbance measured by UV-Vis spectrophotometry. The blank of each pH corresponded to 3.00 ml of respective buffer. The stability was determined in terms of alteration of de maximum peak absorbance.

### *III.4.3. Metals*

Five independent solutions of metals were prepared in buffer at pH 5 with a concentration of 100 µM. To the triplicates of antioxidants solutions at 100µM buffered at pH 5 a volume of metals solutions in a proportion of 1:1 was added.

The stability evaluation in the presence of different metals was analysed over 0, 1 and 2 hours, and 1, 8 and 21 days. These solutions were reserved at 20ºC in dark space with absence of the light.

Aliquots of 3.00 ml were transferred to cuvettes at predetermined time intervals to measure their absorbance by the spectrophotometric method. Metal solutions of each metal were used as blanks.

### *III.4.4. Photostability*

The spectrophotometric measurements were conducted to evaluate the variation in UV/Vis absorbance before and after irradiation. Three independent replicate samples of each substance were prepared and exposed to light providing an overall constant illumination. All samples had a concentration of 100 µM of substance in buffer of pH 5.

Samples of 50 ml of each replicate remained in glass recipient were exposed side-by-side under the lamp with a distance of 42 cm through an appropriate duration of the time. The conditions have been monitored using calibrated radiometers to ensure the specified light exposure is obtained.

The lamp used as light source for photostability testing, emitted an irradiance of 0.62mW/cm2 and 0.74mW/cm2 of UVA and UVB light, respectively, under the test conditions. The UV lamp (Osram, Ultra-Vitalux 300 W) positioned at 54 cm high. The UV lamp was turned on one hour before the experiences, and the UVA and UVB irradiance was measured using a radiometer (Arimed, Cosmedico – Medical Systems, physikalisch UVM-7). The experimental procedure maintained an appropriate control of temperature (20ºC) to minimise the effect of localised temperature changes.

At the end of the exposure period: 5, 10 and 15 minutes, the absorbance spectrum was measured between the wavelengths 200 and 800 nm with a UV/Vis spectrophotometer. The analysis of the exposed samples was performed concomitantly with protected samples used as dark controls. The photostability was qualitatively evaluated by comparison of spectrum of each time of evaluation for each antioxidant.

### *III.5. Compatibility with excipients*

A differential scanning calorimeter (DSC) 200F3 Maia (Netzsh–Gerätebau GmbH) was used for thermal analysis of drugs and excipients. Excipients that were expected to be used in the development of formulation preservatives, surfactants, lipophilic excipients, thickeners, and antioxidants were selected for this study. The selection of excipients was based on their frequency of use in cosmetic formulations, assessed in a previous study (Silva S, 2016).

Thermal analysis is a set of techniques that allows the measurement of changes in physical or chemical properties (and/or its products of reaction) as a function of temperature and/or time, while the substance is subjected to a controlled temperature program (Gill P *et al*. 2010). Thermogravimetry/ derivative thermogravimetry (TG/DTG) and differential scanning calorimetry (DSC) techniques are the most widely distributed and used (Almeida M *et al*. 2010). DSC was used in this work for this purpose.

DSC is a technique which measures the difference in energy supplied to the substance analysed and a thermally inert reference material according to temperature (T), while the substance investigated and the reference are submitted to a controlled temperature program (Almeida M *et al*. 2010; Gill P *et al.* 2010).

The thermo-analytical methods are useful at the pre-formulation stage to obtain information on the physicochemical properties and thermal behaviour of the active substances because they are related to its decomposition (Bezerra G *et al*. 2016). Using the DSC curve, it is possible to measure the difference in energy supplied to the substance and a reference material in function of temperature, both in terms of cooling and heating (Gill P *et al*. 2010; Bezerra G *et al.* 2016). Consequently, the temperature and energy related to these events, including melting point, specific heat, glass transition, crystallization, sublimation, decomposition and liquid crystal transitions, can be evaluated by these techniques (Almeida M *et al.* 2010; Bezerra G *et al.* 2016). Furthermore, data acquired at this stage are extremely important in critical decisions relating to subsequent phases of development. Thermal analysis has shown itself to be an excellent tool for the characterization of the active ingredients and can be used in routine analysis for quality control of cosmetic formulations (Gill P *et al.* 2010).

The physical mixtures between antioxidants and the excipients were prepared in a ratio of 1:1 (W/W), by simply mixing in glass mortar. Some excipients were previously pulverized in porcelain mortar to obtain particle dimensions similar to the antioxidants particles.

Individual samples (drug and excipients) as well as physical mixtures of the drug and selected excipients were weighed directly in the pierced DSC aluminium pan and scanned in the temperature range of 20–400°C under nitrogen atmosphere with flow of 40 ml $min^{-1}$.

A heating rate of 10°C $min^{-1}$was used and the obtained thermograms were observed for any interaction. An empty aluminium pan was used as reference. The onset temperature and melting enthalpy (ΔH) were calculated using Proteus Analysis software.

The DSC cell was calibrated (sensitivity and temperature calibration) with Hg (m.p. -38.8°C), In (m.p. 156.6°C), Sn (m.p. 231.9°C), Bi (m.p. 271.4°C), Zn (m.p. 419.5°C) and CsCl (m.p. 476.0°C) as standards.

### *III.6. Antioxidant activity*

DPPH (2,2'-diphenyl-2-picrylhydrazyl) is characterized as a stable free radical by the delocalization of the odd electron over the molecule as a whole, so that the molecules do not dimerize, like most other free radicals. The delocalization also gives rise to the deep violet colour, with an absorption in ethanol solution at around 517 nm. On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of violet colour. Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is (Burke KE, 2004; Cornacchione S *et al*. 2007; Godic A *et al*. 2014; Karim A, 2004) given by the following equation:

**Equation 1.** DPPH reduction in presence of antioxidant.

$$Z\bullet + AH = ZH + A\bullet$$

Where ZH is the reduced form and A• is free radical produced in the first step.

In DPPH reduction test, antioxidants are able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. Based on this principle, the antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH free radical. The percentage of DPPH discoloration of the sample was calculated according to the equation (Mauludin R *et al*. 2014).

**Equation 2** Calculation of antioxidant activity percentage in DPPH assay.

$$\%\ Antioxidant\ activity = \frac{[Absorbance\ control - Absorbance\ sample]}{Absorbance\ control} x100$$

DPPH method is a rapid, simple, inexpensive and widely used to measure the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity. DPPH method may be utilized in aqueous and nonpolar organic solvents and can be used to examine both hydrophilic and lipophilic antioxidants (Park KT et al. 2011; Perricone N, 1993).

A stock solution of DPPH radical (24 mg/100 ml in ethanol) was prepared. By diluting this solution with ethanol, a working experimental solution with 150 µM was prepared to obtain an absorbance of 0.980 ± 0.02 AU at 517 nm. Based on these, stock solutions of each antioxidant (100 µM) were prepared in ethanol. From these, different dilutions

(0.1–50 µM) were made. The test mixture contained 100 µL DPPH working solution and 100 µL of sample. The reaction 96 well plates were wrapped in aluminium foil and incubated at room temperature (about 25ºC) for 15 minutes in dark. Absorbance of each sample was recorded at 517 nm. For a negative control, 100 µL of ethanol was added in place of antioxidant solution. Rutin was used as a positive control. Inhibition curves were made and $EC_{50}$ values were calculated for all samples by linear regression. Three independent experiments were conducted.

# Chapter IV: Results and Discussion

# Chapter IV: Results and Discussion

## *IV.3.1. Solubility*

### *IV.3.1.1. Solubility in water*

The analysis of water solubility phenomenon is important since this parameter influences the achievement of a desired concentration of active ingredients. This is due to the fact that solubility impacts in extent and rate of permeation of drug, absorption and transportation, and determines pharmacotechnical aspects in formulation development (pre-formulation stage) of adequate topical preparation (Silva JA *et al*. 2010, Bharate S *et al*. 2013).

For this reason, the antioxidants were analysed by the maximum amount of a substance that can be dissolved in a certain volume of solvent, under specific conditions (Savjani KT *et al.* 2012) i.e. by a saturated solution of each compound. Measuring the solubility requires accurate control of temperature and composition in liquid and solid phase (Avdeef A, 2001; Ruenroengklin N *et al*. 2008).

Chemically, the solubility is interpreted by the partition coefficient (Chaurasia G, 2016). The determination of physicochemical properties (pKa, solubility, and log P) facilitates the prediction of pharmacokinetic parameters (absorption, distribution, metabolism and excretion – ADME) of a compound and helps choose an appropriate dosage and form in the process of formulation (Niazi S, 2009; Henchoz Y *et al*. 2009; Stamford NP, 2012, Bharate S *et al*. 2013). Lipophicility is essential physicochemical property impacting ADME behaviour, as it widely contributes to membrane permeation, solubility, protein biding and metabolism (Bharate S *et al*. 2013; Heshmatia N *et al*. 2013).

European Pharmacopeia classifies the solubility regardless of the solvent used, just only in terms of quantification and has defined the criteria as given in Table 8.

**Table 8** Solubility criteriaF.P.(VIII) (2008)

| DESCRIPTIVE TERMS | SOLUBILITY (MG/ML) |
|---|---|
| **VERY SOLUBLE** | **> 1 000** |
| **FREELY SOLUBLE** | **100 – 1 000** |
| **SOLUBLE** | **33 – 100** |
| **SPARINGLY SOLUBLE** | **10 – 33** |
| **SLIGHTLY SOLUBLE** | **1 – 10** |
| **VERY SLIGHTLY SOLUBLE** | **0.1 – 1** |
| **PRACTICALLY INSOLUBLE** | **< 0.1** |

Ascorbic acid and the derivatives were initially evaluated in saturated concentration to understand the best wavelength to maximize the peak absorbance of each. In Figure 10 the aspect of the saturated solutions of AA, AP, MAP, SAP, 3OAA and AA2G in study after rest for stabilization, is depicted. The new compound AA2GS was not used in this study due to the low amount available of this antioxidant.

**Figure 10** Saturated solutions in water of each antioxidant in study at 25ºC. By the following order from left to right: AA2G, AA, 3OAA, MAP, SAP and AP.

The spectra of each analyte at 25ºC is shown in Figure 11 to Figure 16.

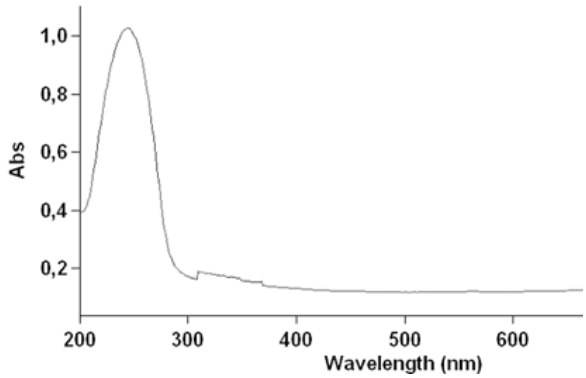


**Figure 11** UV/VIS spectrum of saturated AA concentration at 25ºC.

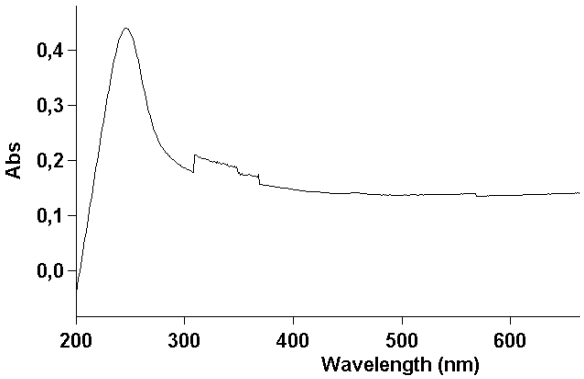


**Figure 12** UV/VIS spectrum of saturated AP concentration at 25ºC.

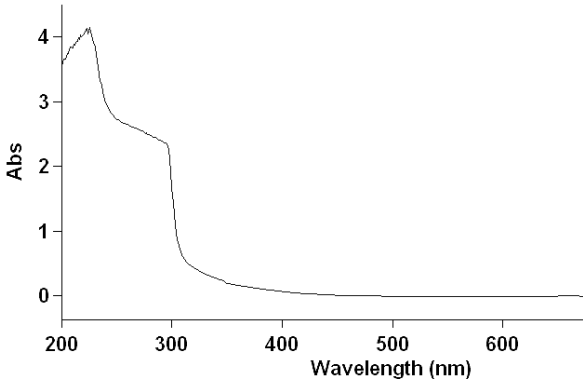


**Figure 13** UV/VIS spectrum of saturated AA2G concentration at 25ºC

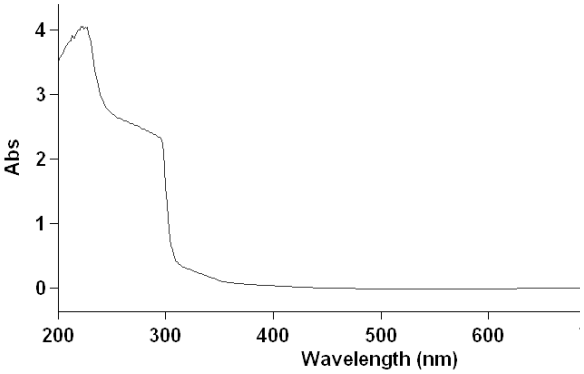


**Figure 14** UV/VIS spectrum of saturated MAP concentration at 25ºC.

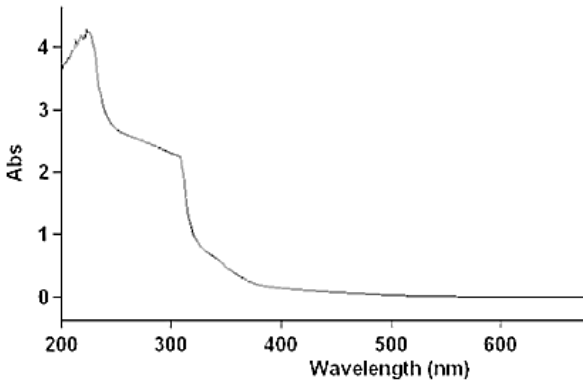


**Figure 15** UV/VIS spectrum of saturated SAP concentration at 25ºC

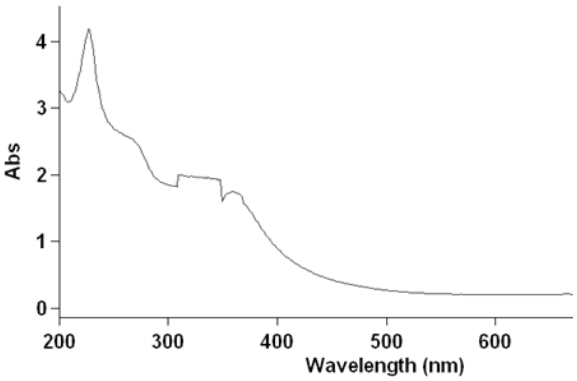


**Figure 16** UV/VIS spectrum of saturated 3OAA concentration at 25ºC.

Based on their spectra, the selected wavelength for solubility studies was 280 nm.

The concentration of each antioxidant against absorbance was plotted to obtain a calibration curve. Antioxidant solutions followed Beer-Lambert's law over focused concentration range at the selected wavelength, with $R^2$ approximately 0.99.

The determinations were altogether in agreement at test temperature (25ºC) by the analytical method employed

Table 9 summarizes the results relative to standard curves obtained for each antioxidant in which the relation between average of absorbance and concentration (g/ml) is linear.

**Table 9** Linearity data for ascorbic acid and its derivatives quantification using a spectrophotometric methodology (280nm)

| Antioxidant | y-Intercept | Slope | Correlation coefficient ($R^2$) | Solubility (g/ml) | SD[a] | CV (%)[a] |
|---|---|---|---|---|---|---|
| AA | 0.1784 | 29095 | 0.999 | 0.30114 | 0.00119 | 0.40 |
| AP | 0.2067 | 5856.6 | 0.999 | 0.00426 | 0.000153 | 3.60 |
| AA2G | 0.3674 | 61250 | 0.9991 | 1.02373 | 0.0123 | 1.20 |
| MAP | 0.3912 | 18.879 | 0.9989 | 0.15736 | 0.000495 | 0.315 |
| SAP | 0.4556 | 7500.6 | 0.9989 | 0.73247 | 0.00288 | 0.395 |
| 3OAA | 0.4084 | 1180.1 | 0.9999 | 2.05610 | 0.00106 | 0.0516 |

[a] The concentration is expressed as the mean and standard deviation of three independent experiments.

Regression analysis suggests a good linearity over the concentration range which is confirmed by a high coefficient of determination ($R^2$). In the present study, the solubility evaluation showed low coefficients of variation (C.V. %).

The absorbance of saturated antioxidant samples (showed in Figure 10) was evaluated at 280 nm and 420 nm, respectively.

Table 10 summarizes the absorbance values at different wavelength and presents the dilution factor applied that allowed the interpolation calculi in linear region of calibration curve.

**Table 10** Comparison the values at ultraviolet and visible light; and, comparison obtained values in experimental analyses with available bibliography data.

| Antioxidant | Absorbance (280nm) | Absorbance (420nm) | Dilution Factor(F) | Saturated concentration (g/ml) 280nm | Bibliography concentration (g/ml) |
|---|---|---|---|---|---|
| AA | 1.0553 | 0.0346 | 10000 | 0.30114 | 0.33 (Rowe, Sheskey et al. 2009) |
| AP | 0.8303 | 0.0070 | 40 | 0.00426 | 0.0056 (Swern 1949, Rowe, Sheskey et al. 2009) |
| AA2G | 0.9944 | 0.0122 | 10000 | 1.02373 | 1.06 (Takeuchi 2006) |
| MAP | 1.1339 | 0.0353 | 4 | 0.15736 | 0.154 (Kameyama K 1996) |
| SAP | 1.0050 | 0.0341 | 10000 | 0.73247 | 0.789 (Aparajita 2014) |
| 3OAA | 1.2172 | 0.0365 | 3000 | 2.05610 | 2.17 (Nikki, McGown et al. 2016) |
| AA2GS[a] | - | - | - | - | Unavailable |

[a] The solubility was not evaluated because of the low stock of this compound.

Therefore, concerning the results, in a first analysis it was possible to conclude that 3OAA and AA2G solubility value in water was higher than 1000 mg/ml at room temperature, being classified according to European Pharmacopeia as “very soluble” and, in its turn, AA, MAP, SAP are classified as “freely soluble” and AP are remarkably the unique “very slightly soluble” of the antioxidants studied.

For AA and its derivatives, the solubility was found to be in the same order of magnitude to the literature data. Slight variations can be justified through influences due to solute-solvent interaction, solid-state interaction as well as ionization state (Smaoui S *et al.* 2013; Smart RC, Crawford CL, 1991). Hence, different pH alters solubility profile with ionisable functional groups (Stamford NP, 2012).

Furthermore, the solubilisation of AA and its derivatives in solvent at room temperature and subsequently heated to obtain complete dissolution of the particles may result in decomposition of a fraction of the compounds. Still, in flask method, the cooling step may involve rapidly cooling of the heated mixture to ambient temperature. This point is highlighted in section of thermal stability. This quick cooling of the mixture might result in the formation of small crystals of antioxidants (Ruenroengklin N *et al*. 2008; Blessy M *et al*. 2014).

### *IV.3.1.2. Solubility in glycerol*

The stability of the active ingredient in the final product is a primary concern to the formulator. Ascorbic acid and its derivatives are, ones more than others, unstable molecules. Although these antioxidants are soluble in water, in this medium they have high propensity to oxidise and/or degrade due to attack by water elements (Tipson R, Horton D,1997; Björklund S *et al*. 2013; Björklund S *et al*. 2010). Accordingly, it is desirable to minimise the use of water to increase the stability of preparations (Björklund S *et al*. 2013; Björklund S *et al*. 2010).

The determination of solubility in glycerine or glycerol for AA and its derivatives was carried out since this solvent is an alternative often used to improve bioavailability of compounds and skin compatibility as well as circumventing the obvious challenges of water solubilisation like prevent hydrolysis(Austria R *et al*. 1997; Bharate S *et al*. 2013; Bissett DL, 2009; Caitlin BS *et al*. 2010; Chaurasia G, 2016).

Table 11 summarizes the results relative to standard curves obtained for each antioxidant in which the relation between average of absorbance and concentration in glycerol (g/ml) is linear.

**Table 11** Linearity data for ascorbic acid and its derivatives quantification using a spectrophotometric methodology (280 nm).

| Antioxidant | y-Intercept | Slope | Correlation coefficient ($R^2$) | Solubility (g/ml) | SD[a] | CV (%)[a] |
|---|---|---|---|---|---|---|
| AA | 1.2261 | 3.4440 | 0.9969 | 0.00898 | 0.000491 | 5.47 |
| AP | 0.4388 | 63.062 | 0.9966 | 0.01112 | 0.000746 | 6.71 |
| AA2G | 0.9430 | 3.0195 | 0.9981 | 0.10424 | 0.00429 | 4.11 |
| MAP | 1.0693 | 1.0198 | 0.9998 | 0.09997 | 0.00358 | 3.58 |
| SAP | 0.5492 | 4.2398 | 0.9994 | 0.11821 | 0.00301 | 2.54 |
| 3OAA | 0.5414 | 3.7397 | 0.9979 | 0.68982 | 0.00741 | 1.07 |
| AA2GS[b] | | | | | | |

[a] The concentration is expressed as the mean and standard deviation of three independent experiments.

[b] The concentration was not calculated because of the low stock of AA2GS

Regression analysis of the calibration data suggests a good linearity over the considered concentration range which is confirmed by a high coefficient of determination ($R^2$). In the present study, the solubility evaluation expresses good precision in repeatability, also known as within-run precision, and was expressed as the coefficient of variation (C.V.%) with good agreement between results of successive measurements obtained under identical conditions.

**Table 12** Comparison between the experimental values of solubility obtained with bibliography data available.

| Antioxidant | Saturated concentration 280nm (g/ml) | Bibliography concentration (g/ml) |
|---|---|---|
| AA | 0.00898 | 0.01 |
| AP | 0.01112 | 0.01 |
| AA2G | 0.10424 | Unavailable |
| MAP | 0.09997 | Unavailable |
| SAP | 0.11821 | 0.132 |
| 3OAA | 0.68982 | Unavailable |
| AA2GS | Not determined | Unavailable |

Even though, the use of glycerol allows an improvement of the stability preventing the oxidation of compounds by minimizing the use of the water, the antioxidants in study demonstrated a lower solubility in this polyol (≈0.1 g/ml) in comparison with water.

Results obtained in this study were similar to the available literature data. The compound that has the higher solubility in glycerol is the SAP.

It can be interesting to solubilize the compounds in preparations with glycerol as co-solvent since it showed to significantly increase the storage stability of oil-in-water emulsions, inhibiting the hydrolysis of AA and derivatives (Ahmad I *et al*. 2011; Lavecchia R, Zuorro A, 2015). Data on the solubility of AA and its derivatives in glycerol is scarcely available. However, the study of this property is important in the sense that glycerol is a versatile excipient in semi-solid formulations used in pharmaceutical preparations as a means of improving smoothness, providing

lubrication and as a humectant. Glycerol is a hygroscopic substance which keeps the preparation moist (Björklund S *et al*. 2010; Cornacchione S *et al.* 2007; Coronado MH *et al.* 2015; Rossi M, 2008).

Glycerol is furthermore a component of natural moisturizing factor (NMF) that is naturally present in skin and protects from severe drying. And, for this reason glycerol is used in commercial skin care lotions and creams where the beneficial function of these compounds is ascribed to their hygroscopic properties (Nakazawa H *et al.* 2012; Nayama S *et al*. 1999; Obonga W *et al*. 2013). Yet, it is clear that barrier function as well as the mechanical properties of stratum corneum do not only depend on its water content, but more important, on the state and molecular organization of non-aqueous stratum corneum lipid and protein components (Björklund S *et al*. 2010, Obonga W *et al*. 2013). Moreover, glycerol can enhance the skin penetration. The explanation for the effect includes the interaction of glycerol with intercellular lipids, the inhibition of the lipid transformation by glycerol, the desmolytic effect of glycerol, and the hydrating effect of glycerol (Baertschi S, 2006; Bettinger J *et al*. 1998).

### *IV.3.2. Stability*

Physical and chemical stability of pharmaceutical or cosmetic ingredients is a matter of great concern as it affects the safety and efficacy of the drug or cosmetic product. The Food and Drug Administration (FDA) and International Conference in Harmonisation (ICH) guidance state the requirement of stability testing data to understand how the quality of drug substance and drug product changes with time under the influence of various environmental factors (Blessy M *et al*. 2014; Roy L, Urooj A, 2013)

The stability studies are carried out to achieve several purposes such as establishment of the degradation pathway of the active and final products, differentiate degradation products that are related to the active ingredient, determination of intrinsic stability of a drug substance in formulation, reveal the degradation mechanism such as hydrolysis, oxidation, thermolysis or photolysis of the active ingredient or the product (Reynolds D *et al*. 2002) understand the chemical properties of molecules, to generate more stable formulations, to produce a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions and to solve stability-related problems (Brummer H, 2011; Roy L, Urooj A, 2013).

### *IV.3.2.1. pH*

Hydrolysis reactions are often pH dependent are catalysed by either hydronium ion or hydroxide ions (specific-acid or specific-base catalysis, respectively) (Blessy M *et al*. 2014; Sui X *et al.* 2014).

The most important parts of chemical stability are performances on accelerated testing and kinetics of pH profiles (Baertschi S, 2006). To test hydrolysis, all antioxidants were submitted under a range of pH buffers to know what is the pH where each one is more stable. As far as the effectiveness of the cosmetic formulations is concerned, the pH is often regarded as a significant parameter. The pH of human skin normally ranges from 4.5 to 6.0. Therefore, in order for a formulation to possibly gain admission for industrial application, it should have a pH that is close to this range (Silva GM, Maia Campos PM, 2000; Maier H *et al*. 2005; Masaki H, 2010; Puvabanditsin P, Vongtongsri R, 2016).

The graphs depicting absorbance versus time at different times (0, 1, 2 hours, 1, 8, 21 days) are overlapped for each analyte in Figures 17 to 23, in order to understand the variation in the absorbance peak for each antioxidant molecule.

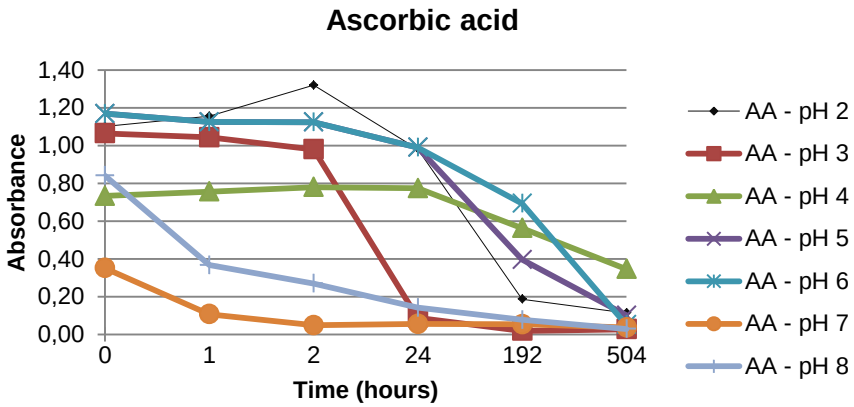


**Figure 177** The relative AA stability given by variation in pH buffers over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) at 25ºC.

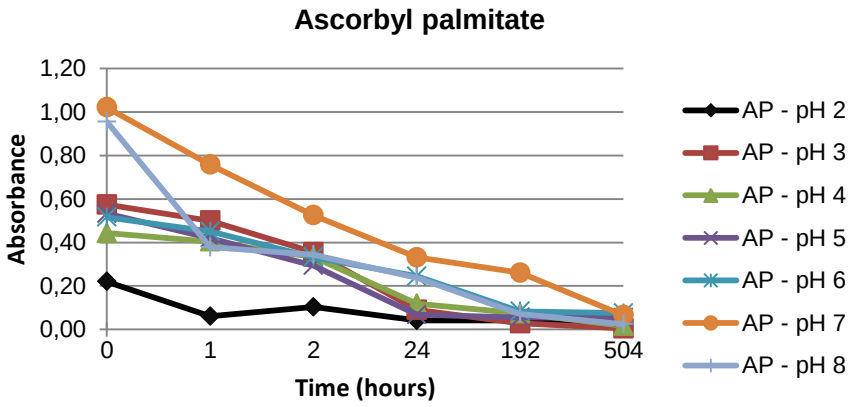


**Figure 188** The relative AP stability given by variation in pH buffers over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) at 25ºC.

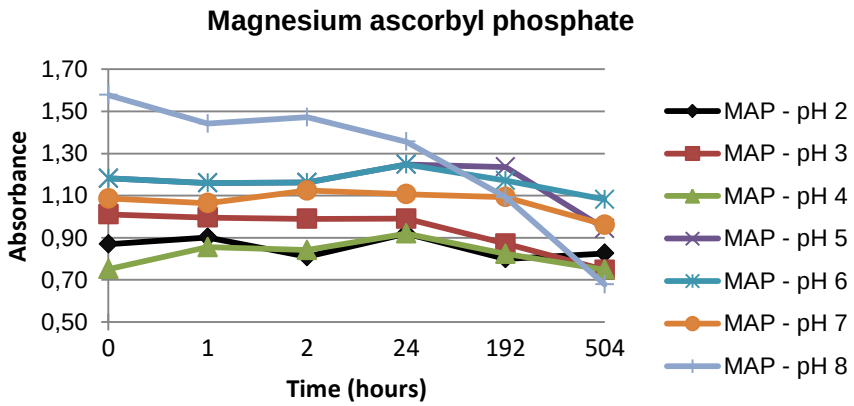


**Figure 1919** The relative MAP stability given by variation in pH buffers over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) at 25ºC.

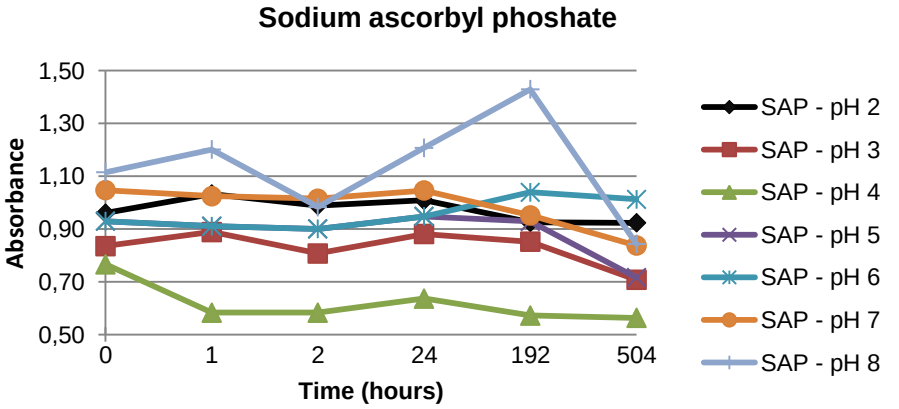


**Figure 200** The relative SAP stability given by variation in pH buffers over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) at 25ºC.

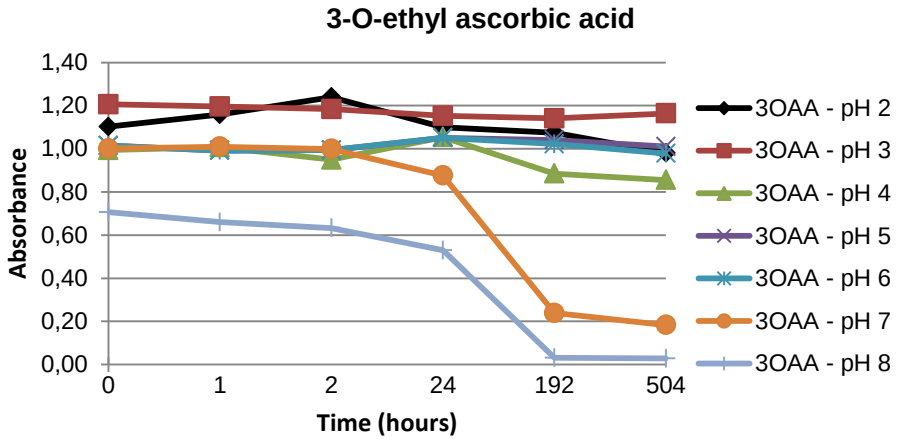


**Figure 221** The relative 3OAA stability given by variation in pH buffers over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) at 25ºC.

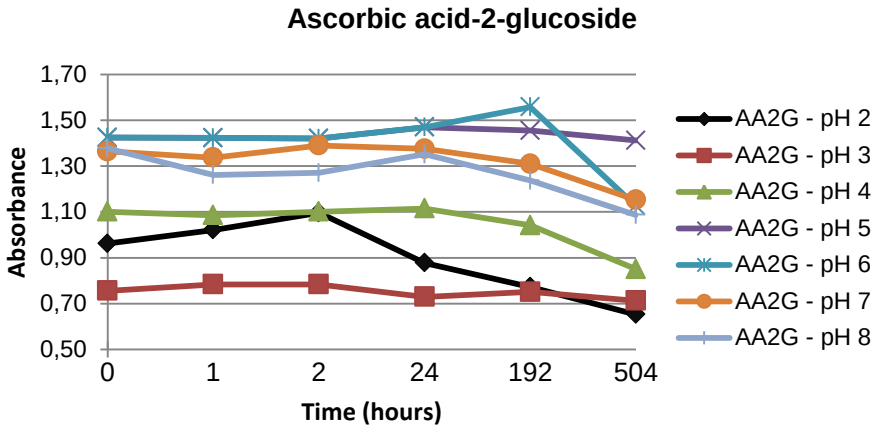


**Figure 232** The relative AA2G stability given by variation in pH buffers over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) at 25ºC.

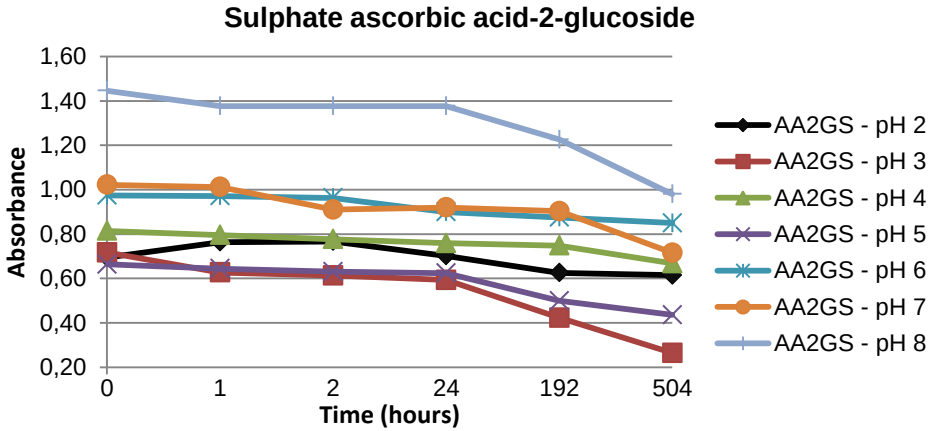


**Figure 213** The relative AA2GS stability given by variation in pH buffers over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) at 25ºC.

In order to categorize the stability of antioxidants in different conditions a criterion was created to categorize the data depicted in

Table **13**. In this characterisation, the compounds stability is divided in high stability, stable, poor stability and unstable categories. Variations in three aspects of the spectra were considered:

i) hyperchromic or hypochromic effects: recorded an increase of peak absorbance or a decrease of absorbance respectively;

ii) bathochromic or hypsochromic shifts: recorded a change of spectral band position in the absorption spectrum of a molecule to a longer wavelength (lower frequency), or a shorter wavelength (higher frequency), correspondingly;

iii) appearance of new peaks, meaning instability of antioxidants

**Table 13** Stability classification criteria of antioxidants in coordination with tested variants.

| Classification | Symbol | Hypochromic/ Hyperchromic | Bathochromic/ Hipsochromic | New peaks |
|---|---|---|---|---|
| **High Stability** | HS | 0-5% | 0 % | No |
| **Stable** | S | 5-20% | 0-1 % | No |
| **Poor stability** | PS | > 20% | 2-5 % | No |
| **Unstable** | U | > 20% | >5 % | Yes (1 or more) |

Table 14 summarizes the classification of the experimentally results in agreement with the previous criteria. This have the intention of simplify the comprehension of stability and instability over the studied periods of each pH buffered antioxidant solution.

**Table 14** Stability over time of AA, AP, MAP, SAP, 3OAA, AA2G and AA2GS in different buffered solution at pH 2, 3, 4, 5, 6, 7 and 8.

| Antioxidant | Time (hours) | pH 2 | pH 3 | pH 4 | pH 5 | pH 6 | pH 7 | pH 8 |
|---|---|---|---|---|---|---|---|---|
| AA | 1 | HS | HS | S | S | S | PS | PS |
| | 2 | S | S | S | S | PS | PS | PS |
| | 24 | S | PS | S | S | S | PS | PS |
| | 192 | PS | U | PS | S | PS | U | U |
| | 502 | U | U | PS | U | PS | U | U |
| AP | 1 | PS | S | S | PS | S | PS | PS |
| | 2 | PS | PS | PS | PS | PS | PS | PS |
| | 24 | PS | PS | PS | PS | PS | PS | PS |
| | 192 | PS | PS | PS | U | PS | U | U |
| | 502 | PS | U | PS | U | PS | U | U |
| MAP | 1 | HS | S | S | HS | S | HS | S |
| | 2 | S | PS | S | HS | PS | HS | S |
| | 24 | S | S | PS | S | S | HS | S |
| | 192 | S | S | S | U | S | U | U |
| | 502 | S | PS | HS | U | S | U | U |
| SAP | 1 | S | S | PS | HS | S | HS | S |
| | 2 | HS | PS | PS | HS | PS | HS | S |
| | 24 | S | U | S | HS | S | HS | S |
| | 192 | S | U | PS | U | S | U | U |
| | 502 | S | U | PS | U | S | U | U |
| 3OAA | 1 | S | HS | HS | HS | S | HS | S |
| | 2 | S | HS | HS | HS | PS | HS | S |
| | 24 | S | HS | S | HS | S | S | PS |
| | 192 | HS | PS | S | HS | S | U | U |
| | 502 | S | S | S | HS | S | U | U |
| AA2G | 1 | S | PS | HS | HS | S | HS | S |
| | 2 | S | S | HS | HS | PS | HS | S |
| | 24 | S | PS | HS | HS | S | HS | HS |
| | 192 | S | PS | S | U | S | U | U |
| | 502 | PS | PS | PS | U | PS | U | U |
| AA2GS | 1 | S | S | PS | HS | S | HS | HS |
| | 2 | S | S | PS | S | PS | S | HS |
| | 24 | HS | S | PS | S | S | S | HS |
| | 192 | S | PS | PS | U | S | U | U |
| | 502 | S | PS | PS | U | S | U | U |

Considering the results, in the pH range of 4 to 5 the decomposition is controlled, while at pH 7 to 8 it increases. These reactions of AA degradation occur quickly in basic conditions and the compound degrades itself irreversibly in a biologically inactive form (2,3-diketo-L-gulonic acid) (Austria R *et al*. 1997) .Thus, the stability of ascorbic acid is higher in an acidic medium. Therefore, the AA solutions are stable in pH which are below the pKa of AA itself (pKa 4.2), thus reducing the charge density on the molecule.

AA instability is due to its unique 1-one-2,3-diol-2-ene set of connected functional group. In the 1-one-2,3-diol-2-ene system, the 3-OH group ionizes first at acidic pKa of 4.17. Thus at physiological pH, AA is 99.9% ionized to its highly polar, water soluble 3-monoanion. AA loses one electron that gives radical and following looses another electron to generate the di-radical, which rearranges to the inactive dehydroascorbic acid. In the process, AA gives up two protons and two electrons to quench free radicals being formed in solution (Hacisekvi A, 2009; Stamford NP, 2012; Nikki T *et al.* 2016).

Measurement data of ascorbyl 2-phosphates: SAP and MAP salts in these conditions shows that neutral or basic solutions guarantee the highest stability, whereas in acid solutions these have been described to easily hydrolyse to AA and inorganic phosphate AA (Stamford NP, 2012; Nikki T *et al.* 2016). However from the depicted results is evident that these are more stable in range of pH than AA.

In turn, ascorbyl palmitate AP was found to be stable at acidic pH similar to AA as supported by a previous study (Mauludin R *et al*. 2014). The definitive differences in behaviour between the derivatives were evident: SAP and MAP kept their stability for a long period of time (about 192 hours of storage in the dark at room temperature), whereas AP already showed instability after 24 hours. This can be explained by the fact that esterification at the 6 position of AP does not prevent hydrolysis of the molecule (Austria R *et al*. 1997; Segal A, Moyano A, 2008). It is reasonable to believe that the introduction of the phosphate group in 2-position protects the enediol system of the molecule from hydrolysis better than esterification in 6 position with long lipophilic chains.

The 3OAA antioxidant has the highest stability at pH 5 along the study period. In broadly pH's showed to be stable, or highly stable in the first day, following with a hypso/hyperchromic alteration after this time. These data are in accordance with previously evidences that showed that 3-O ethyl derivative prevents the ionization of 62

the 3-OH, effectively stabilizing the 1-one-2,3-diol-2-ene system against oxidation (Hong J *et al*. 2004; Hurtado N *et al*.. 2009; Jin S, Miao X, 2008).

AA2G and AA2GS are the derivatives that are more stable in broadly pH values. The first is stable for 1 month in pH 5. The new derivative is especially stable in alkaline pH. The 2-O-phosphate derivatives although does not mask the 3-OH group, also stabilizes the 3-OH group to ionization and hence its subsequent rapid oxidation, not by masking it but by suppressing its ionization by the introduction of the even more acidic P(=O)=OH group (Brummer H, 2011; Burke KE, 2004; Cadenas E, Packer L, 2007; Caitlin BS *et al*. 2010; Nikki T *et al.* 2016).

The AA2G and AA2G derivatives shows improve the physicochemical properties under pH influence, these takes advantage of the fact that hydrolysis of a functional group in the promoiety attached to a functional group in the parent molecule (AA), presents like a prodrugs stabilized forms (Stamford NP, 2012).

The rate of hydrolysis depends also of the temperature (described in the next section). Improper pH ranks with exposure to elevated temperature as a factor most likely to cause a clinically significant loss of drug, resulting from hydrolysis and oxidation reactions (Sui X *et al*. 2014).

### *IV.3.2.2. Temperature*

The triplicate spectra of absorbance for each antioxidant solution at the period of time analysed were in agreement at each tested temperature. The solutions were buffered at pH 5, the pH where the majority of antioxidants involved demonstrated being stable. The spectra traces at different times (0, 1, 2 hours, 1, 8, 21 days) are schematized in Figure 27 to Figure 30 to understand the temperature at which each molecule is most stable.

Similar to pH stability, Table 15 summarizes the categories found in agreement to the classification criteria presented in

Table **13**.

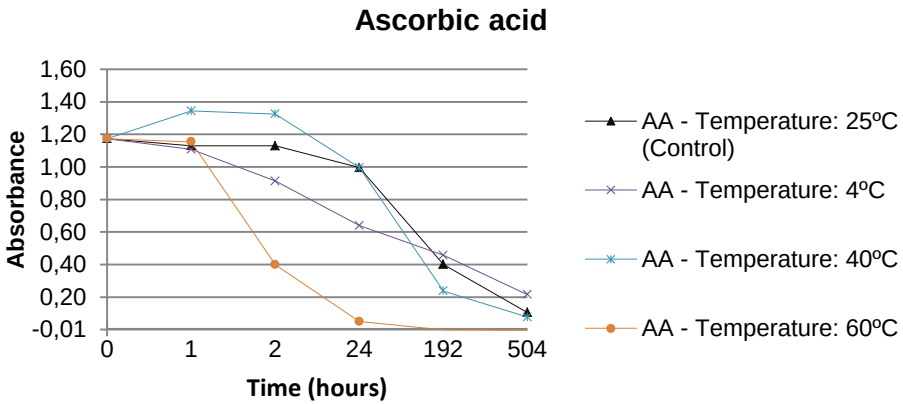


**Figure 244** The relative AA stability given by variation in temperature over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) buffered at pH 5.

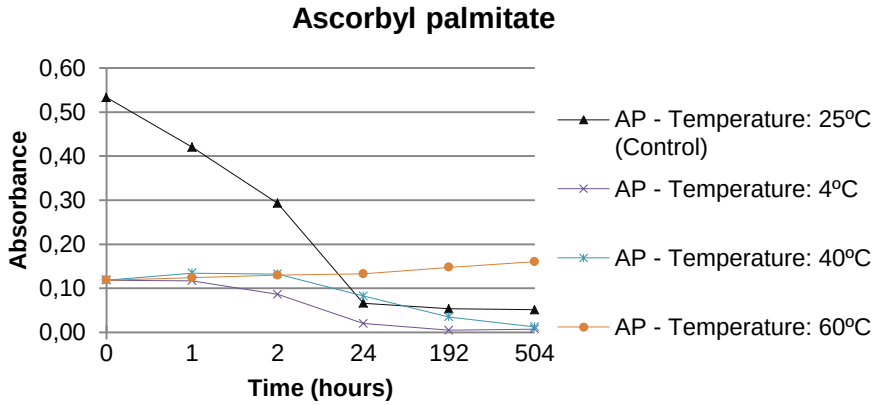


**Figure 255** The relative AP stability given by variation in temperature over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) buffered at pH 5.

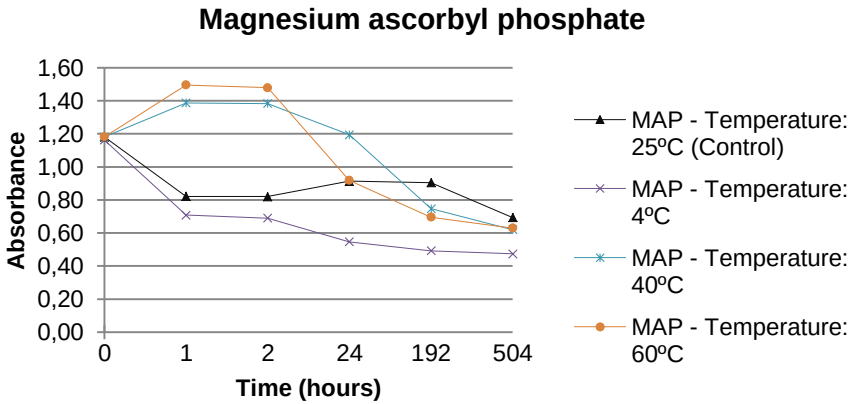


**Figure 266** The relative MAP stability given by variation in temperature over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) buffered at pH 5

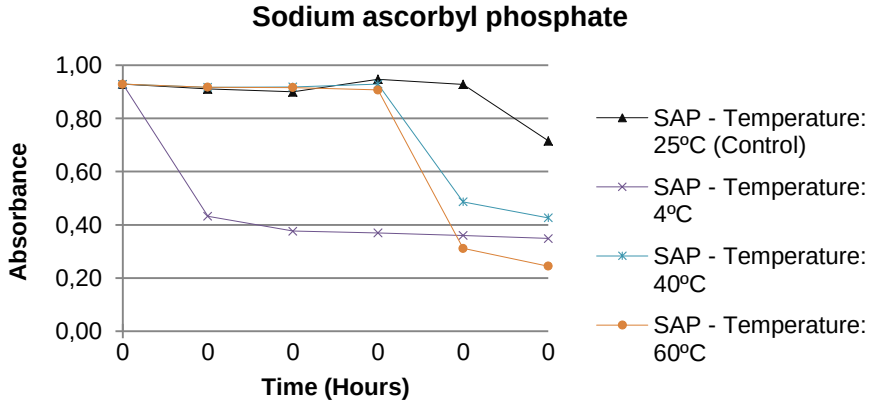


**Figure 277** The relative SAP stability given by variation in temperature over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) buffered at pH 5

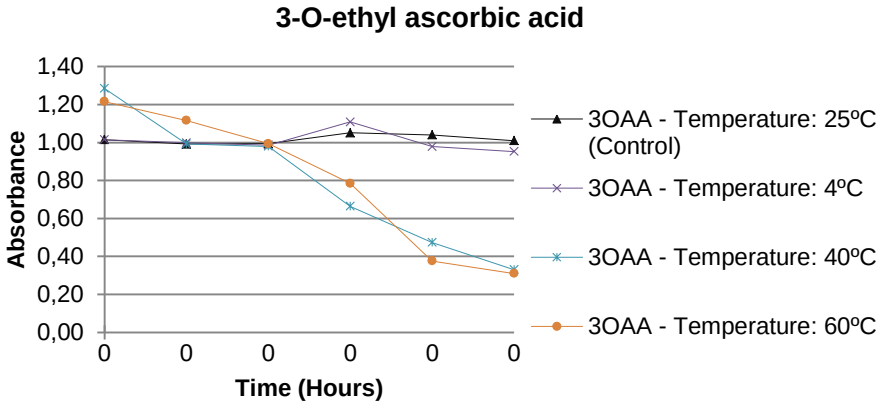


**Figure 288** The relative 3OAA stability given by variation in temperature over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) buffered at

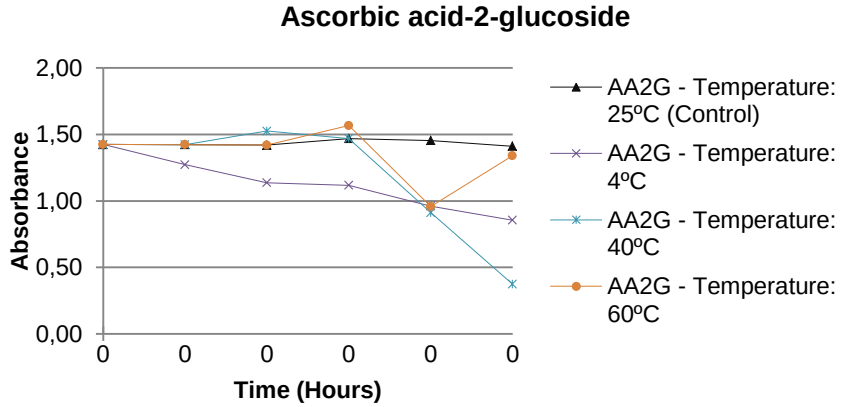


**Figure 2929** The relative AA2G stability given by variation in temperature over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) buffered at pH 5.

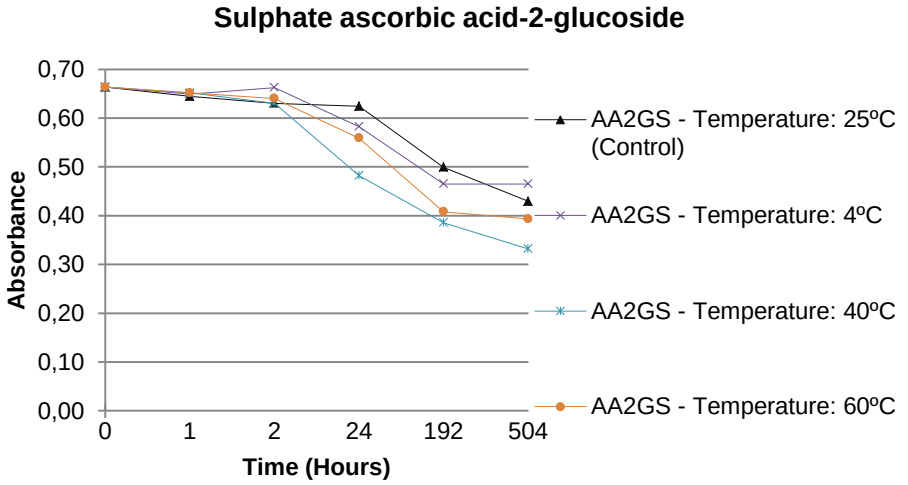


**Figure 300** The relative AA2GS stability given by variation in temperature over the time of analysis (0, 1, 2 hours and 1, 8 and 21 days) buffered at pH 5.

**Table 15** Stability over the time of AA, AP, MAP, SAP, 3OAA, AA2G and AA2GS buffered solution at 5 at 4, 25, 40 and 60ºC.

| Antioxidant | Time (hours) | 4ºC | 25ºC | 40ºC | 60ºC |
|---|---|---|---|---|---|
| AA | 1 | S | HS | S | HS |
| | 2 | PS | HS | PS | PS |
| | 24 | PS | S | S | PS |
| | 192 | PS | PS | PS | U |
| | 502 | PS | U | PS | U |
| AP | 1 | HS | PS | S | HS |
| | 2 | PS | PS | S | S |
| | 24 | PS | PS | PS | S |
| | 192 | PS | U | PS | PS |
| | 502 | PS | PS | PS | PS |
| MAP | 1 | PS | HS | PS | PS |
| | 2 | PS | HS | PS | PS |
| | 24 | PS | S | PS | S |
| | 192 | PS | HS | S | S |
| | 502 | PS | HS | PS | PS |
| SAP | 1 | PS | HS | HS | HS |
| | 2 | PS | HS | HS | HS |
| | 24 | PS | HS | HS | HS |
| | 192 | PS | S | HS | PS |
| | 502 | PS | PS | HS | PS |
| 3OAA | 1 | HS | HS | PS | S |
| | 2 | HS | HS | PS | S |
| | 24 | S | HS | PS | PS |
| | 192 | HS | HS | PS | PS |
| | 502 | S | HS | PS | PS |
| AA2G | 1 | S | HS | HS | HS |
| | 2 | PS | HS | S | HS |
| | 24 | PS | HS | S | S |
| | 192 | PS | HS | PS | PS |
| | 502 | PS | HS | PS | S |
| AA2GS | 1 | HS | PS | PS | PS |
| | 2 | HS | PS | PS | PS |
| | 24 | S | PS | PS | PS |
| | 192 | PS | PS | PS | PS |
| | 502 | PS | PS | PS | PS |

The results show that AA decomposes with the time at different temperatures. Data demonstrate that concentration of AA is more constant at room temperature followed by refrigerated conditions (Telang P, 2013; Sui X *et al*. 2014). Storage under refrigeration minimizes AA degradation at long period whereas storage at room temperature encourages significant degradation with time in comparison with other storage conditions studied. The findings of this experiment showed that AA is an unstable chemical compound that is degraded when exposed to elevated temperatures. At high temperatures 40ºC and 60ºC an increase of absorbance is noted and a slow diminishing of its colour that pointed a possible accelerated degradation of AA into dehydroascorbic acid since this degradation product is responsible for the increase of the solution colour (Telang P, 2013; Sui X *et al*. 2014).

The marked differences in behaviour between the derivatives were evident: SAP and MAP kept their stability to nearly 70-80% even after 8 days of storage in the dark at ambient temperature, whereas AP already showed great instability after the same period and at the same temperature. Even so, SAP is more stable than MAP in the long-term studies for all temperatures. MAP experienced only slight concentration losses after storage at room temperature and at 4°C and 25ºC, and an increase of absorbance at 40ºC and 60ºC.

In first hours of analysis at 60ºC AA2G and AA2GS are stable compounds. But, at the end of the time of study period its degradation rates being approximately of 50%. Thus, AA2G exhibited a high stability with it degradation rates being insignificant at room temperature up until 21 days. AA2GS showed high stability conserved at 4ºC. 3OAA showed the highest stability at 4ºC and 25ºC. The compound absorbance diminishes after 1 day of storage at 40ºC and 60ºC.

As patent in results, temperature is one of the most important factors affecting antioxidant stability. Generally, heating causes an acceleration of the initiation reactions, and hence a decrease in the activity of antioxidants (Ruenroengklin N *et al*. 2008; Sadineni V *et al.* 2014). And this thermal variation may change the mechanism of action of some antioxidants (Sui X *et al*. 2014). Most reactions are faster at higher temperatures than lower temperatures. The speed of a reaction duplicates for each 10ºC increase in temperature (Hurtado N *et al*. 2009).

Therefore thermal analysis is crucial to establish the stability of each molecule or formulation. It is understandable that temperature is an important factor and its influence can be minimized by selecting an appropriate storage temperature (Hurtado

N *et al*. 2009). And it can affect particular reactions in which antioxidants participate (mainly the reactions with lipid radicals compared to side reactions, in which the tested compounds do not act as antioxidants or act as pro-oxidants) (Roy L, Urooj A, 2013).

#### *V.3.2.3. Metals*

The antioxidant solutions were prepared at pH 5 with different metal solutions and analysed at different periods of time. The results at different times (0, 1, 2 hours, 1, 8, 21 days) were overlapped in order to understand de variation in the absorbance peak for each antioxidant molecule.

From this spectrum's was summarized in next figures Figure 31 to Figure 35.

In order to simply the stability evaluation of each antioxidant in presence of metals, Table 16 summarizes the categories found for the experimental results, in agreement to the classification criteria depicted in Table 13.

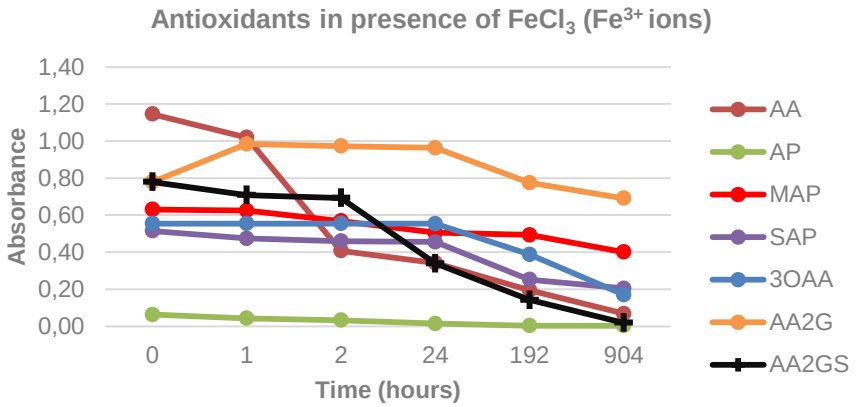


**Figure 31** Antioxidants solutions in presence of 50 µM $FeCl_3$ solution ($Fe^{3+}$) at different periods of time (0, 1 and 2 hours and 1, 8 and 21 days).

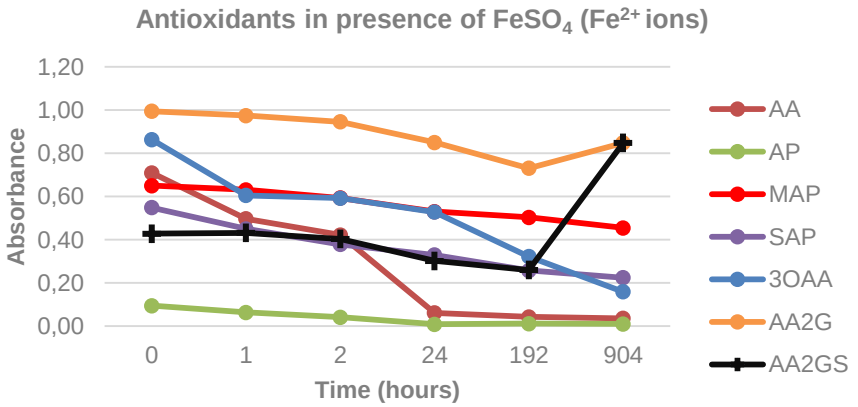


**Figure 32** Antioxidants solutions in presence of 50 µM $FeSO_4$ solution ($Fe^{3+}$) at different periods of time (0, 1 and 2 hours and 1, 8 and 21 days).

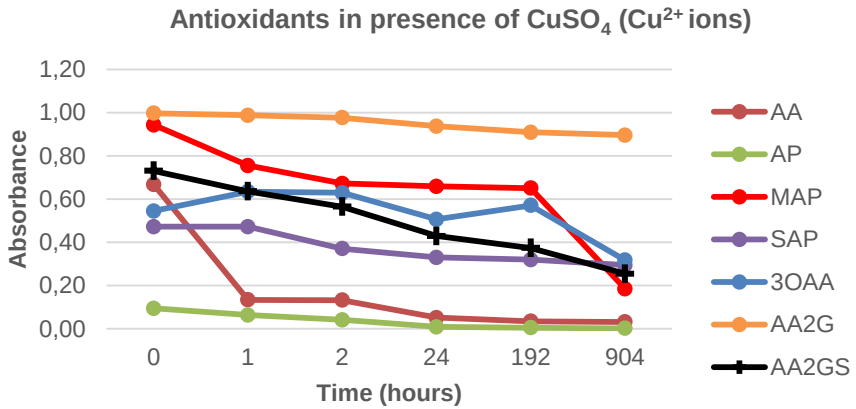


**Figure 33** Antioxidants solutions in presence of 50 µM $CuSO_4$ solution ($Cu^{2+}$) at different periods of time (0, 1 and 2 hours and 1, 8 and 21 days).

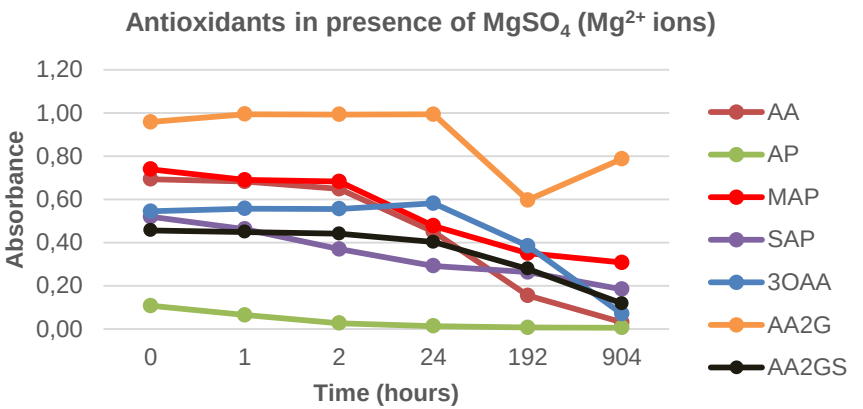


**Figure 34** Antioxidants solutions in presence of 50 µM $MgSO_4$ ($Mg^{2+}$) at different periods of time (0, 1 and 2 hours and 1, 8 and 21 days).

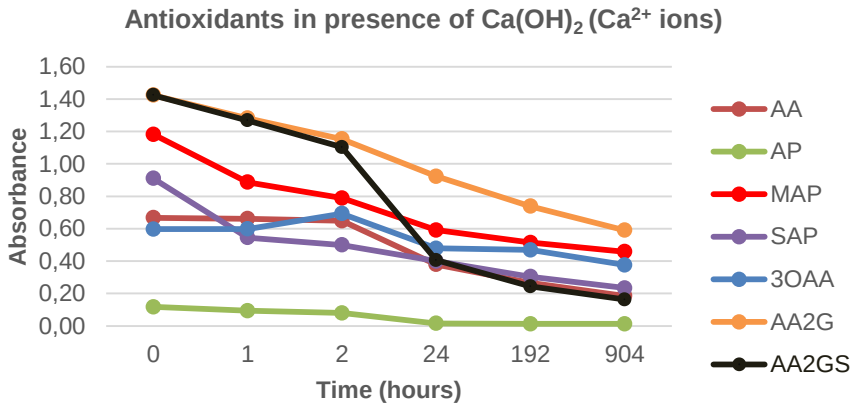


**Figure 315** Antioxidants solutions in presence of 50 µM $Ca(OH)_2$ solution ($Ca^{2+}$) at different periods of time (0, 1 and 2 hours and 1, 8 and 21 days).

**Table 16** Summary of antioxidants solutions in presence of metallic solutions at different times of measurement.

| Antioxidant | Time (hours) | Fe(III) | Fe(II) | Cu(II) | Mg(II) | Ca(II) |
|---|---|---|---|---|---|---|
| AA | 1 | S | PS | PS | S | HS |
| | 2 | PS | PS | PS | S | HS |
| | 24 | PS | PS | PS | PS | PS |
| | 192 | PS | PS | PS | PS | PS |
| | 502 | PS | PS | PS | PS | PS |
| AP | 1 | S | PS | PS | PS | PS |
| | 2 | PS | PS | PS | PS | PS |
| | 24 | PS | PS | PS | PS | PS |
| | 192 | PS | PS | PS | PS | PS |
| | 502 | PS | PS | PS | U | U |
| MAP | 1 | HS | HS | S | S | PS |
| | 2 | S | S | PS | S | PS |
| | 24 | S | S | PS | PS | PS |
| | 192 | PS | PS | PS | PS | PS |
| | 502 | PS | PS | PS | PS | PS |
| SAP | 1 | S | S | HS | S | PS |
| | 2 | S | PS | PS | PS | PS |
| | 24 | S | PS | PS | PS | PS |
| | 192 | PS | PS | PS | PS | PS |
| | 502 | PS | PS | PS | PS | PS |
| 3OAA | 1 | HS | PS | S | HS | HS |
| | 2 | HS | PS | S | HS | S |
| | 24 | PS | PS | S | S | PS |
| | 192 | PS | PS | HS | PS | PS |
| | 502 | PS | PS | HS | PS | PS |
| AA2G | 1 | S | S | HS | HS | HS |
| | 2 | S | S | HS | HS | HS |
| | 24 | PS | PS | S | HS | HS |
| | 192 | PS | PS | PS | PS | PS |
| | 502 | PS | PS | S | PS | S |
| AA2GS | 1 | S | HS | PS | PS | PS |
| | 2 | PS | HS | PS | PS | PS |
| | 24 | PS | S | PS | PS | PS |
| | 192 | PS | S | PS | PS | PS |
| | 502 | PS | S | PS | PS | PS |

The analysis combines the metals $Cu^{2+}$, $Fe^{2+}$, Fe3+, $Mg^{2+}$ and $Ca^{2+}$ with AA and its derivatives.

Addition of Fe(II), Fe(III), Cu(II) and Ca(II) ions to 100µM AA solution decrease its stability in opposite to Mg(II). The levels of AA were lower in presence of $Cu^{2+}$, $Fe^{2+}$, $Fe^{3+}$ and $Ca^{2+}$ levels. This is in agreement with the fact that the pro-oxidant activity of AA arises from its ability to reduce transition metals (e.g. $Fe^{3+}$ or $Cu^{2+}$) by a one-electron transfer mechanism. This results from the fact that AA can act as a monodentate ligand that reacts with metal cations in aqueous solutions resulting in low stability water-soluble complexes. This complexation reaction involves the lactone ring and the side chain. Subsequently, in the presence of catalytic amounts of metal ion it is rapidly oxidised to dehydroascorbate (Burke KE, 2004; Masaki H, 2010; Nimse S, Pal D, 2015; Oroian M *et al.* 2015). So, trace levels of transition metals can participate in the metal-catalyzed Haber-Weiss reaction (superoxide-driven Fenton reaction) as well as catalyze the oxidation of AA (Lynch SM *et al.* 1996; Nimse S, Pal D, 2015).

In presence of $Mg^{2+}$ and $Ca^{2+}$ AA concentration does not suffer a significantly decrease. These findings suggest that AA have a good stability in first hours in presence of these metals.

Accordingly, the results found that all the tested metal ions had an effect on the absorbance of AA and its derivatives. The addition of metallic ions to antioxidant compounds resulted in slight changes of the absorbance spectrum with bathochromic shifts from the original bands. The complex formed in presence of ions, highlights the potential of AA to chelate metals (Hong J *et al*. 2004; Dolińska B *et al*. 2012; Cidade H *et al*. 2017).

The AP is oxidised by transition metal ions presented in traces, being the most unstable, similar to previous reports in which metals showed to affect the concentration and the bioavailability of this antioxidant to exert its scavenging activity (Almeida M *et al*. 2010; Mauludin R *et al*. 2014).

The stability of compounds MAP, SAP and 3OAA against an oxidative environment was measured in the presence of metals. As shown in previous table, these compounds exhibited their higher stability in the presence of $Fe^{2+}$ and $Fe^{3+}$ ions as 90% was maintained even after 24 h whereas AA was almost completely degraded. Between of MAP, SAP and 3OAA, the compound 3OAA was more stable than AA and MAP and SAP except in presence of $Fe^{3+}$ ion here its stability was relatively less than MAP and SAP.

Most of the investigations pointed out that 2-OH and 3-OH groups play the main role in the stability of AA. The results report that different AA derivatives modified on different OH groups by simple alkyl groups present an increased stability in presence of metals $Fe^{2+}$ and $Fe^{3+}$ (Hong J *et al*. 2004; Dolińska B *et al*. 2012).

AA2G showed much higher stability against $Ca^{2+}$, $Cu^{2+}$ and $Mg^{2+}$ degradation than AA. In fact, only 5% of was degraded in the presence of 100 μM of metals. This stability suggests that the glycosyl sugar groups attached to ascorbic acid inhibited the oxidation catalysed by these metals. Hence, the glycosyl group introduced into C-2 of ascorbic acid was proposed to sterically hinder the access or binding of metal ion to AA (Bae HK *et al*. 2002; Hong J *et al*. 2004; Dolińska B *et al*. 2012). The AA2GS accompanies the behaviour of AA2G in first hours of analysis, but in presence of $Ca^{2+}$, $Cu^{2+}$, $Fe^{3+}$ and $Mg^{2+}$ suffered an accentuated decrease in absorbance. This phenomenon only not occur in presence of $Fe^{2+}$ here the stability of AA2GS is preserved through 21 days of study.

Transition metals exhibit different oxidation states and can interact with a number of negatively charged molecules (Sadineni V *et al*. 2014) and may be present in a number of excipients and, therefore, these may catalyse the oxidations of active ingredients (Snape T *et al*. 2010; Spiclin P *et al.* 2013), resulting in oxidation-reduction reactions that compromises the stability of final product (Snape T *et al*. 2010; Spiclin P *et al.* 2013). Adding chelating agents to water to sequester heavy metals and working in special manufacturing equipment (e.g. glass) are some means used to reduce the influence of heavy metals on a formulation (Hong J *et al*. 2004; Hovorka S, Schöneich C, 2001).

### *IV.3.3. Photostability*

Photolytic degradation can be an important limiting factor in the stability of active ingredients of cosmetics, especially molecules as antioxidants that are very susceptible to oxidation (Caitlin BS *et al*. 2010). A compound can be affected chemically by radiation of a wavelength only if it absorbs radiation at that wavelength and the energy exceeds a threshold (Maier H *et al*. 2005; Baertschi S, 2006). Exposure to, primarily, UV illumination may cause oxidation and scission/photolysis of covalent bonds. In susceptible compounds photochemical energy can create free radical intermediates, which can perpetuate chain reactions (Burke KE, 2004; Ahmad I *et al*. 2011). Light stress conditions can induce photo-oxidation by free radical mechanism. Functional groups like carbonyls, nitro aromatic, N-oxide, alkenes, aryl chlorides, weak C-H and O-H bonds, sulphides and polyenes are likely to promote photosensitivity (Burke 2004, Caitlin BS *et al*. 2010; Blessy M *et al*. 2014).

Photostability refers to determination of the stability of substances in presence of ultraviolet radiation. UV light generates ROS which produce some harmful effects on the skin including photo-carcinoma and photo-aging. To combat these problems, topical antioxidants should be resistant to light degradation in order to exert their action (Segal A, Moyano A, 2008; Sheraz M *et al*. 2011; Blessy M *et al*. 2014).

Irradiation conditions (lamp distance and irradiation time) were established in order to put in evidence the photo-instability of AA. The AA derivatives were exposed to the same conditions for comparison purposes. The curves at different times were overlapped in order to understand the variations in the absorption spectrum.

Figure 36 to Figure 42 show the results of photostability obtained for each antioxidant.

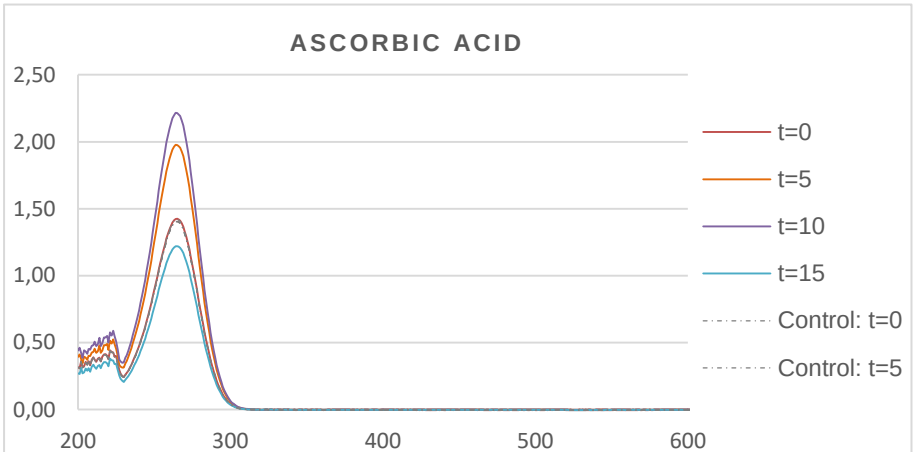


**Figure 36** Photostability of AA at 0, 5, 10 and 15 minutes under exposure of UV light.

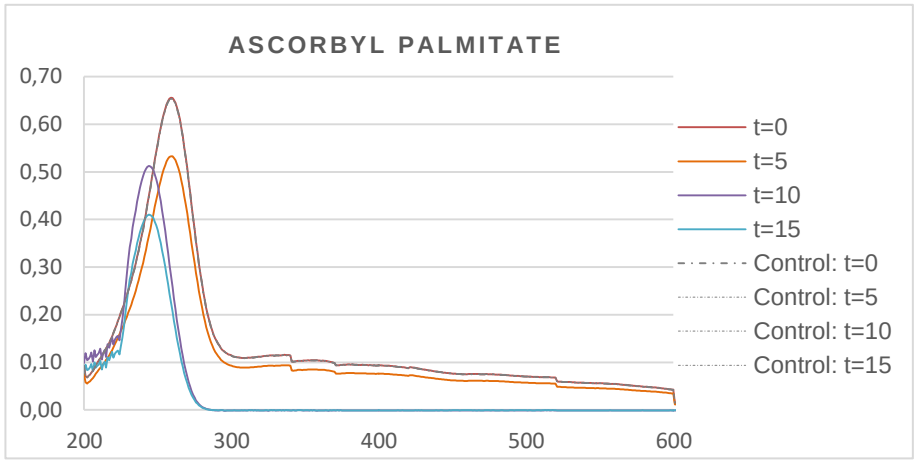


**Figure 37** Photostability of AP at 0, 5, 10 and 15 minutes under exposure of UV light.

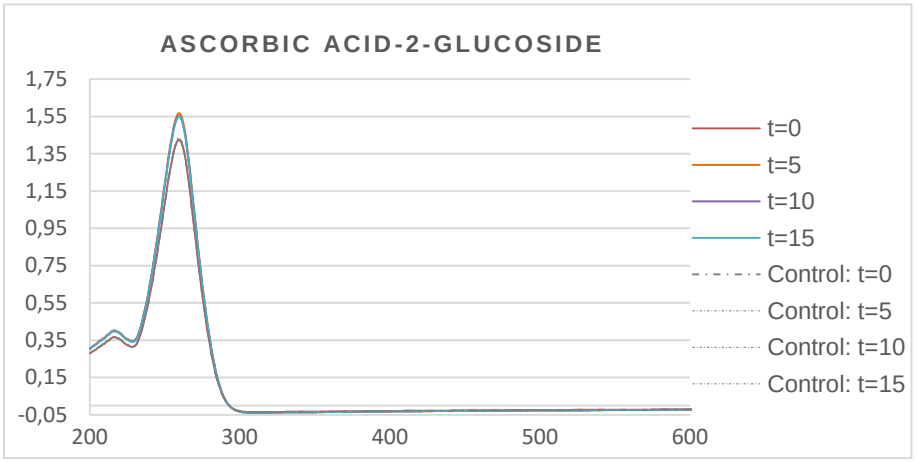


**Figure 38** Photostability of AA2G at 0, 5, 10 and 15 minutes under exposure of UV light.

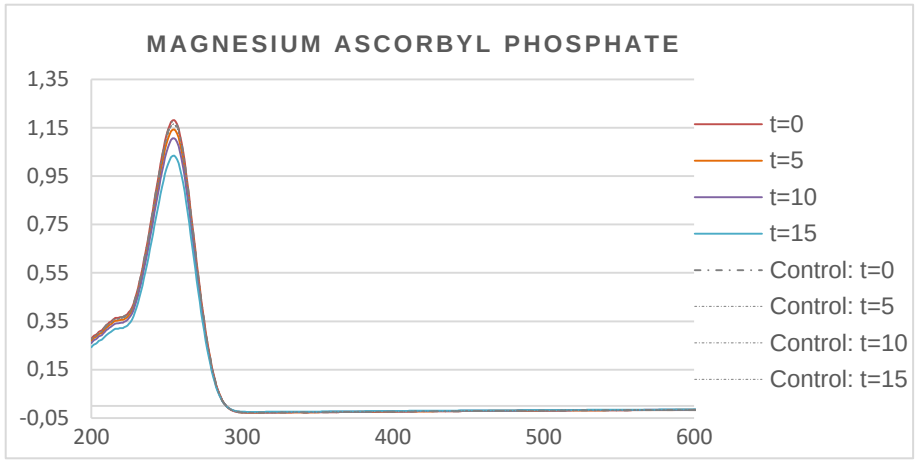


**Figure 39** Photostability of MAP at 0, 5, 10 and 15 minutes under exposure of UV light.

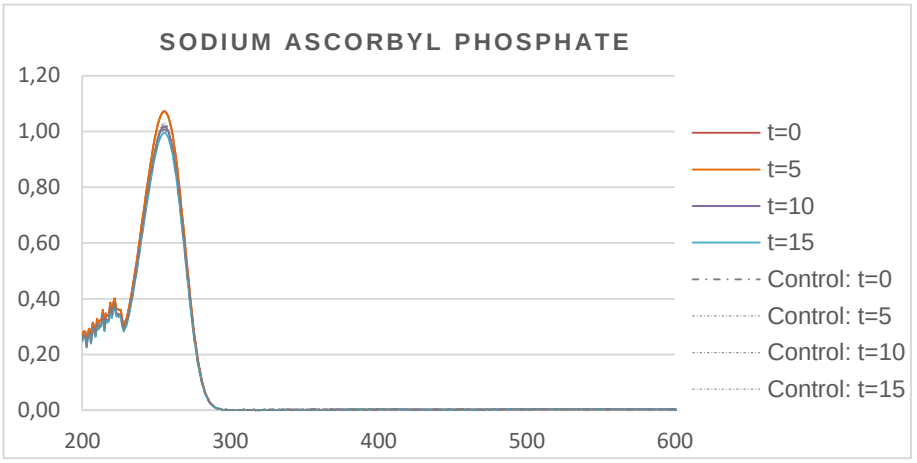


**Figure 40** Photostability of SAP at 0, 5, 10 and 15 minutes under exposure of UV light.

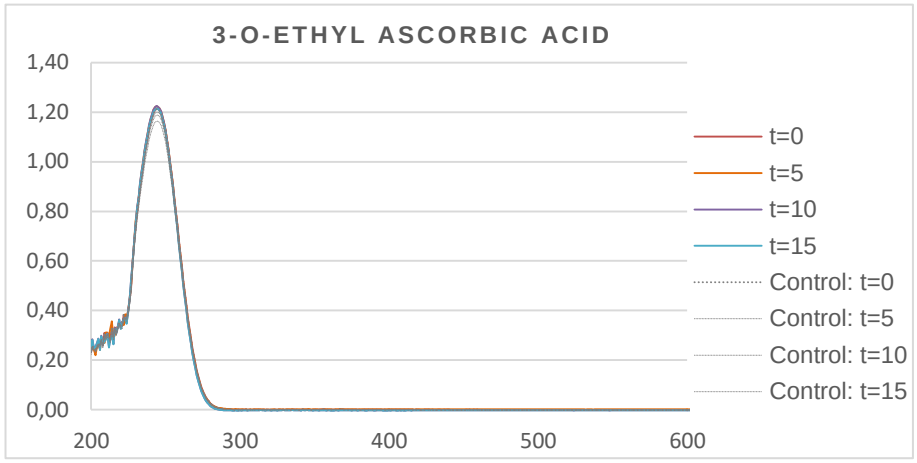


**Figure 41** Photostability of 3OAA at 0, 5, 10 and 15 minutes under exposure of UV light.

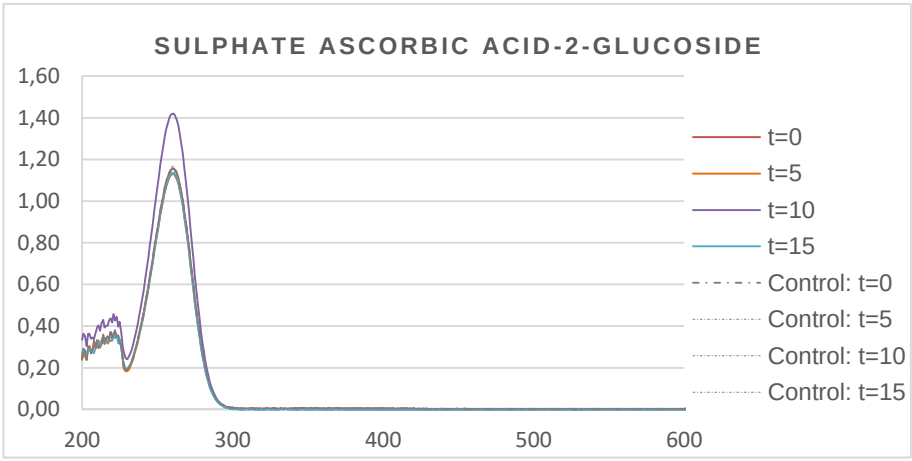


**Figure 42** Photostability of AA2GS at 0, 5, 10 and 15 minutes under exposure of UV light.

As antioxidants and active ingredients in anti-aging cosmetics, AA, AP, MAP, SAP, 3OAA, AA2G protect skin by neutralizing ROS generated on exposure to sunlight. So, to exert their action, these antioxidants should be stable under sun exposure.

AA and AP are the antioxidants that demonstrated more instability in the presence of UV light. The absorbance of AA decreases, translated by hipsochromic phenomenons, and, AP spectra show also bathochromic variations. The other antioxidants demonstrate good stability in all periods of testing. AA2GS, the new derivative, showed an increase in absorbance after 10 minutes of exposure followed by a significant decrease in absorbance after 15 minutes.

The increased photodegradation of AA was previously reported to be related with possible concomitant increases in dehydroascorbic acid (DHA) and 2,3-diketogulonic acid levels (Tikekar RV *et al*. 2011; Telang P, 2013). Studies showed that ascorbyl radical formation occurs simultaneously with AA degradation. Consistent with these studies, UV treatment was shown to accelerate dark storage degradation, with AA radicals continued to be formed after an initial UV treatment (Tikekar RV *et al.* 2011).

Like AA, AP is itself biologically active. The photo instability of AP may be responsible for damaging effects that probably outweighs the benefits of its antioxidant potential. This evidence was discussed in previous studies that showed that after topical treatment, AP was not as effective as AA in providing protection against UVB radiation exposure in mouse models (Ochiai Y *et al.* 2006), although when applied to porcine skin did provide protection against UV-induced free radicals. However, another study in human keratinocyte cell culture has suggested that despite its antioxidant properties, AP may potentiate skin damage following UVB irradiation (Meves A *et al*. 2012).

The phosphate esters of AA and AA2G, as stable forms in presence of UV light suggest that these antioxidants are good actives against photo-damage induced by radiation. SAP and MAP were shown to have a protective effect due to the maintenance of a normal AA level by conversion of SAP and MAP to AA in skin tissue (Kobayashi S *et al.* 1996; Miyai E *et al.* 1997; Nayama S *et al*. 1999). Relatively to the stable derivative – AA2G, its photostability suggests it can be effective to prevent solar radiation-mediated deleterious effects. Miyai *et al.* (1997) defend that AA2G reduced UV damage of human skin keratinocytes and fibroblasts more effectively than ascorbyl 2-phosphate salts because in their study a photo protective effect against UVB was demonstrated. Photostability of these derivatives should be further studied using solar simulated radiation that mimics real sunlight exposure.

### *IV.3.4. Compatibility with excipients of topical formulations*

According to many authors, an incompatibility phenomenon comes from chemical reactions between the antioxidants and excipient that can lead to the active ingredient's degradation resulting in new degradation products or impurities that can change quality, safety and efficacy of pharmaceutical or cosmetic formulations (Bezerra G *et al*. 2016; Gill P *et al*. 2010).

The energy is introduced simultaneously into a sample cell (which contains the sample of antioxidant, excipient or the mixture of either) and a reference cell (containing only the solvent). Temperatures of both cells are raised identically over time. The difference in the input energy required to match the temperature of the sample to that of the reference would be the amount of excess heat absorbed or released by the molecule in the sample (during an endothermic or exothermic process, respectively). As a result of the presence of the molecule of interest, more energy is required to bring the sample to the same temperature as the reference (Gill P *et al*. 2010; Neves AR *et al.* 2013).

In order to establish the comparison and evaluate the results from the mixtures with excipients, the Table 17 summarizes the melting point and function of each antioxidant and excipient.

**Table 17** Raw materials in compatibility study between antioxidants and excipients.

| Raw material | Sample | Melting point (ºC) | Function | Bibliography |
|---|---|---|---|---|
| Active ingredient | AA | 190ºC | Antioxidant | (Valko 2007, SpecialChem 2017) |
| | AP | 116º | | (Swern 1949, Meves 2002) |
| | MAP | >300ºC | | (Tagawa and Tabata 1988, Kameyama K 1996) |
| | SAP | N/A | | |
| | 3OAA | 111-116ºC | | (Jina 2008, Shalmashi 2008, Tai 2014) |
| | AA2G | 158-163ºC | | (Yamamoto and Muto 1992) |
| | AA2GS | N/A | | - |
| Excipients | AEE | 59.4 to 59.8 °C | Surfactant agent and stabilizer for w/o as well as for o/w emulsions. | (SpecialChem 2016) |
| | HEC | 140ºC | Film Former, thickeners and Stabilizers. Gelling properties | (Chemical 2015) |
| | TP | 2.5-2.5ºC | Antioxidant with synergistic effect with AA | (Puyabanditsin and Vongtongsri 2006) |
| | EDTA | 237ºC | Chelating agent | (Gill 2010) |
| | CP | 12.5ºC | Gelling agent, viscosity increasing agent | (Gill 2010) |
| | MIG | 0ºC | Triglycerides of the fractionated plant fatty acids C8 and C10, oil phase, emollient | (Cremer 2017) |
| | PCP | 166ºC | Emulsifier oil in water | (SpecialChem 2017) |

N/A – Not information available

The next figures Figure 43 to Figure 50 shows the antioxidant curves of DSC in the heating rate of 10ºC $min^{-1}$. DSC curves are in agreement in the three replicates. For this reason only one curve of each is depicted. The Table 18 summarizes the thermoanalytical parameters obtained.

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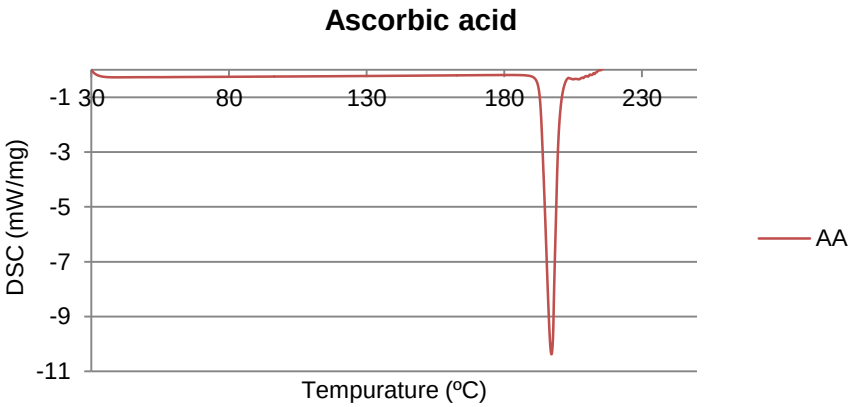


**Figure 43** Ascorbic acid curve of DSC in the heating rate of 10ºC $min^{-1}$.

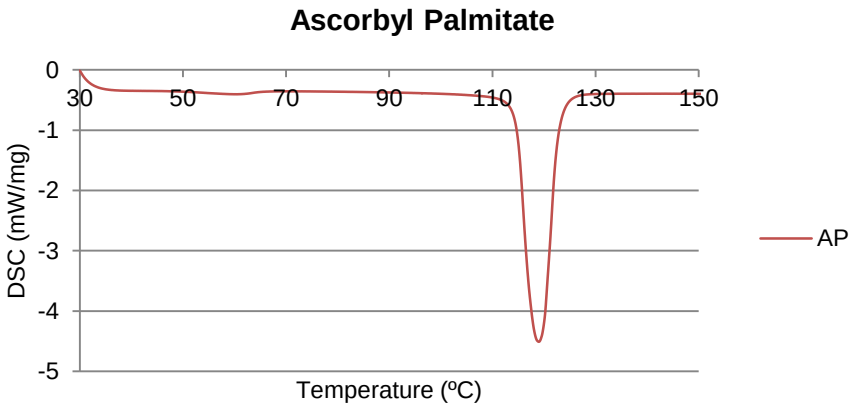


**Figure 44** Ascorbyl palmitate curve of DSC in the heating rate of 10ºC $min^{-1}$.

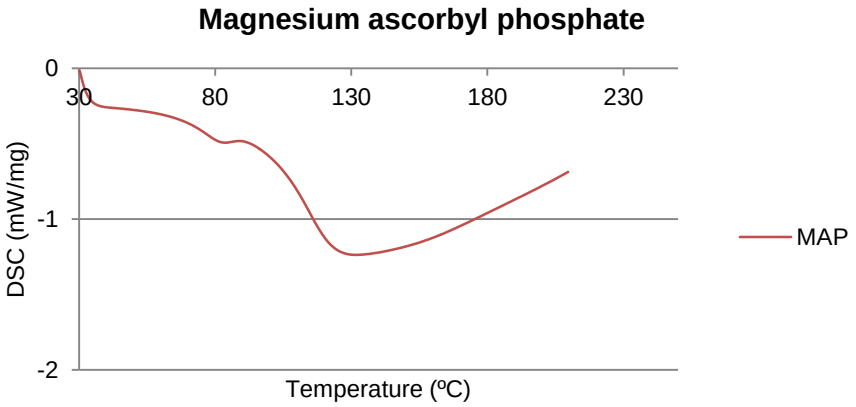


**Figure 45** Magnesium ascorbyl acid curve of DSC in the heating rate of 10ºC $min^{-1}$.

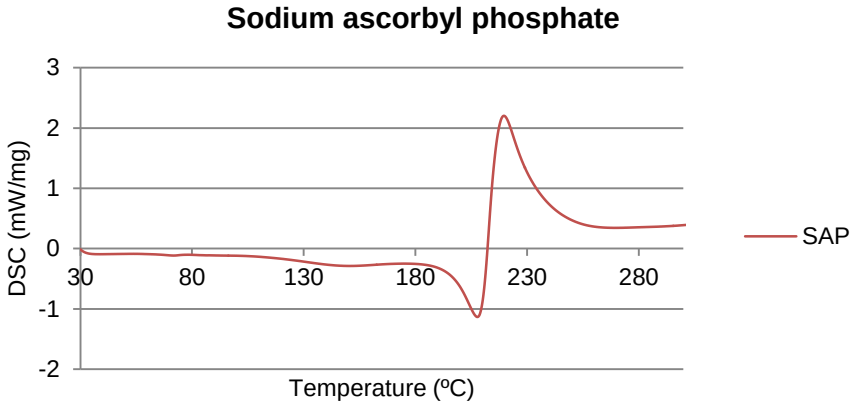


**Figure 46** Sodium ascorbyl phosphate acid curve of DSC in the heating rate of 10ºC $min^{-1}$.

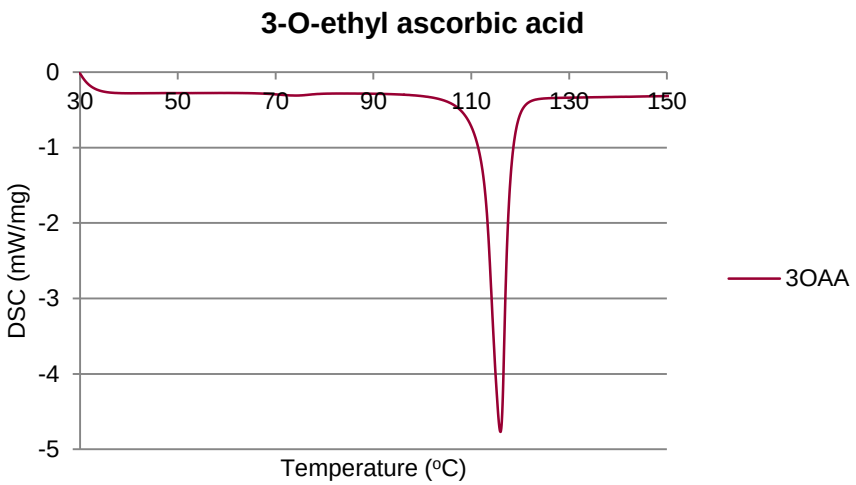


**Figure 47** 3-O-ethyl ascorbic acid curve of DSC in the heating rate of 10ºC $min^{-1}$.

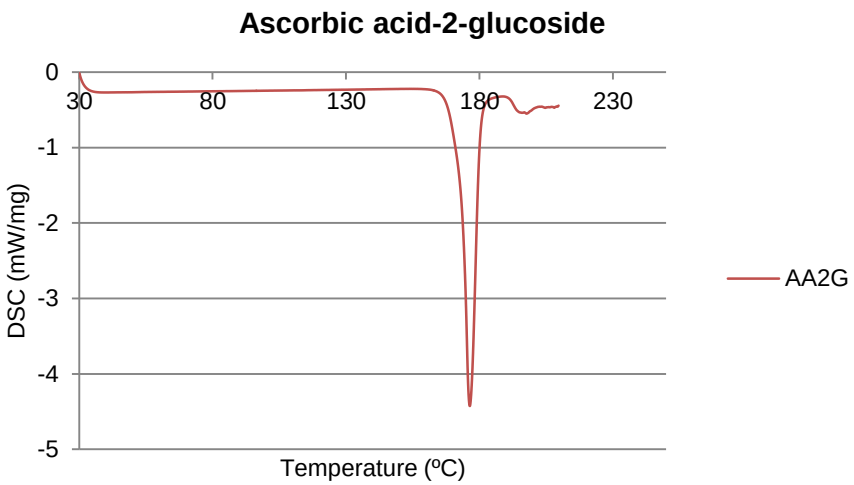


**Figure 48** Ascorbic acid-2-glucoside acid curve of DSC in the heating rate of 10ºC $min^{-1}$.

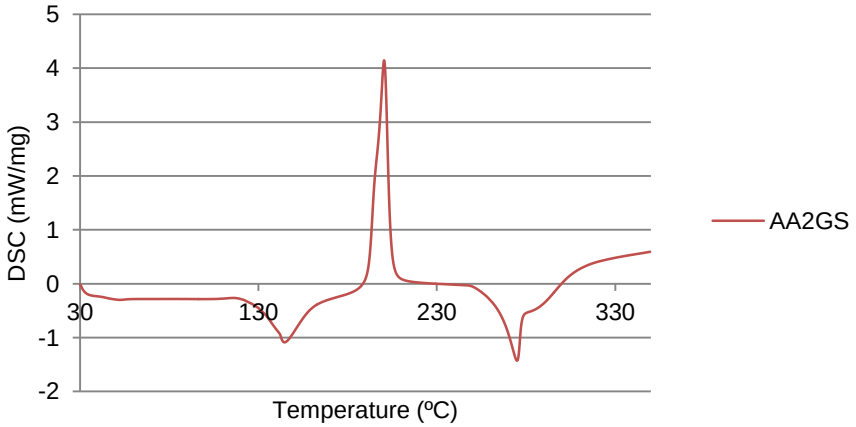


**Figure 49** Sulphate ascorbic acid-2-glucoside curve of DSC in the heating rate of 10ºC $min^{-1}$.

**Table 18** DSC thermoanalytical data of antioxidant in the heating rate of 10ºC $min^{-1}$.

| Isolated antioxidants | Event | $T_{onset}$/ºC | $T_{peak}$/ºC | ΔH J $g^{-1}$ |
|---|---|---|---|---|
| AA | Exo | 192.9 | 197.1 | -328.6 |
| AP | Exo | 114.7 | 119.0 | -137.9 |
| MAP | Exo | 105.6 | 127.0 | -66.12 |
| SAP | Exo | 196.3 | 207.6 | -266.3 |
| 3OAA | Exo | 110.0 | 115.1 | -108.3 |
| AA2G | Exo | 173.0 | 176.4 | -139.8 |
| AA2GS | Endo | 195.0 | 200.5 | 223.1 |

The thermograms of excipients obtained in the same conditions and the result of binary mixtures of each antioxidant with an excipient (ratio 1:1 W/W) are shown in the next figures: Figure 50 – Figure 98.

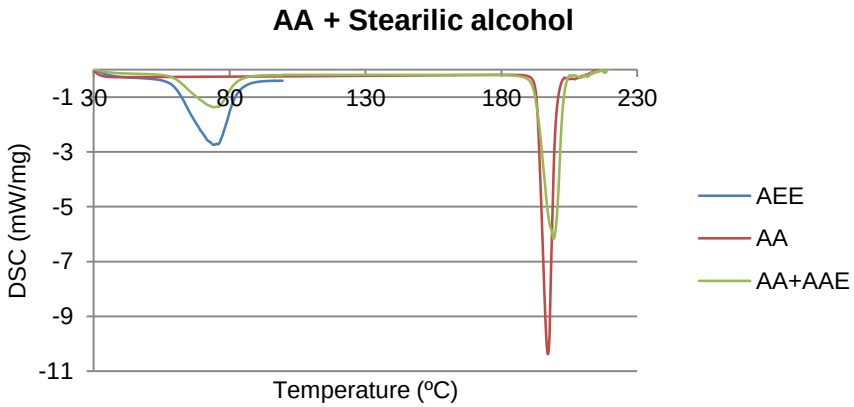


**Figure 50** AA + AEE curve of DSC in the heating rate of 10ºc $min^{-1}$.

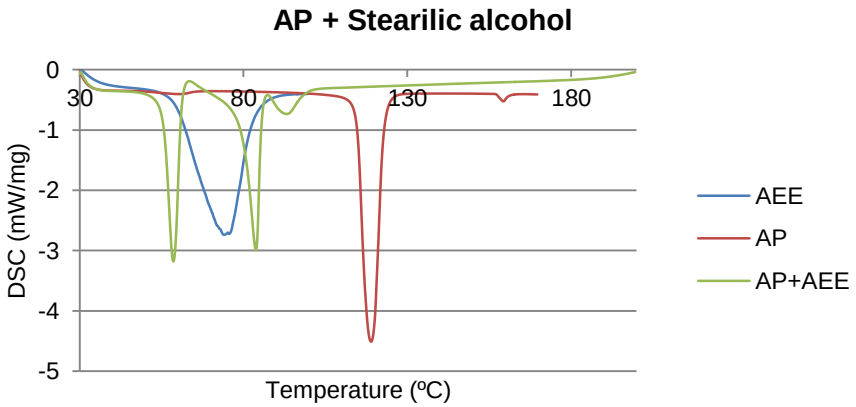


**Figure 51** AP + AEE curve of DSC in the heating rate of 10ºC $min^{-1}$

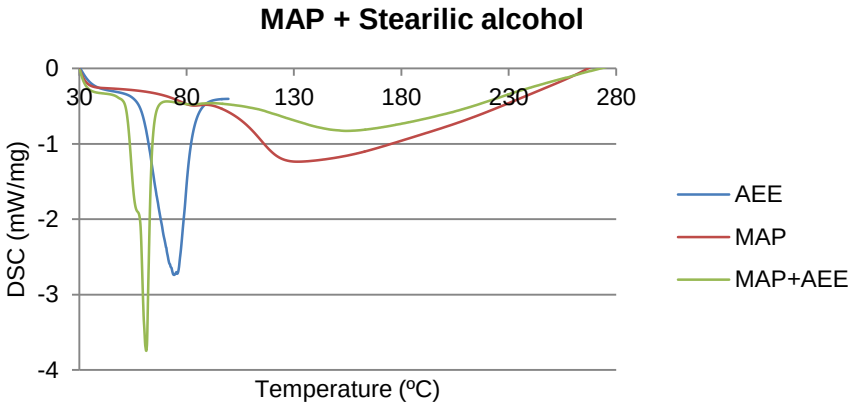


**Figure 52** MAP + AEE curve of DSC in the heating rate of 10ºC $min^{-1}$

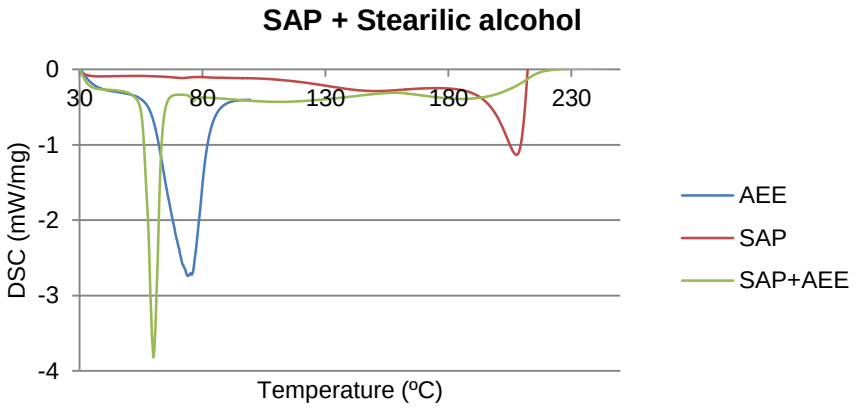


**Figure 53** SAP + AEE curve of DSC in the heating rate of 10ºC $min^{-1}$

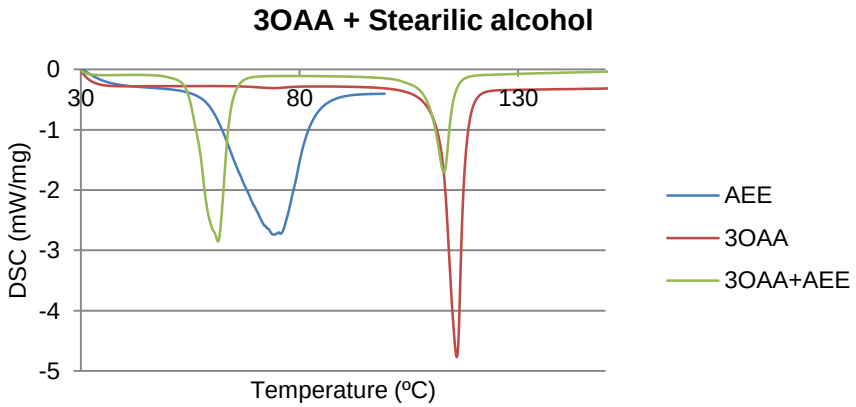


**Figure 54** 3OAA + AEE curve of DSC in the heating rate of 10ºC $min^{-1}$

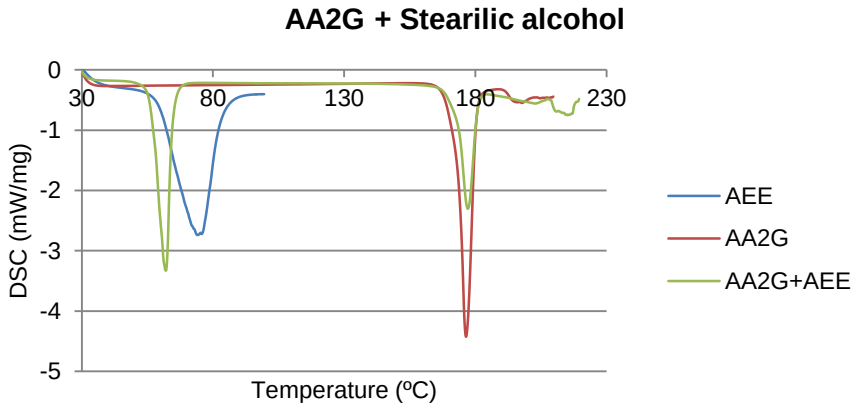


**Figure 55** AA2G + AEE curve of DSC in the heating rate of 10ºC $min^{-1}$

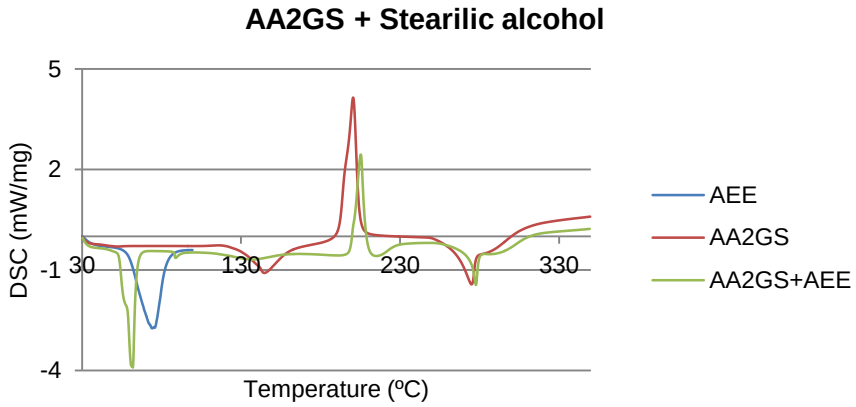


**Figure 56** AA2G + AEE curve of DSC in the heating rate of 10ºC $min^{-1}$

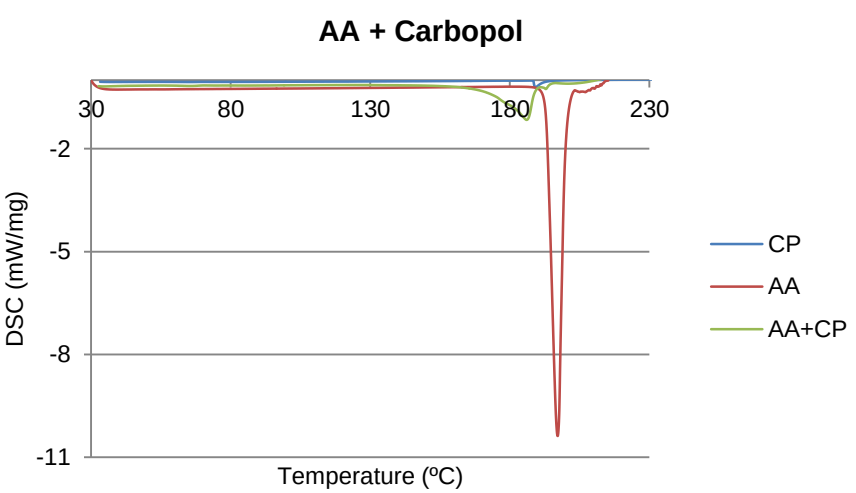


**Figure 57** AA + CP curve of DSC in the heating rate of 10ºC $min^{-1}$

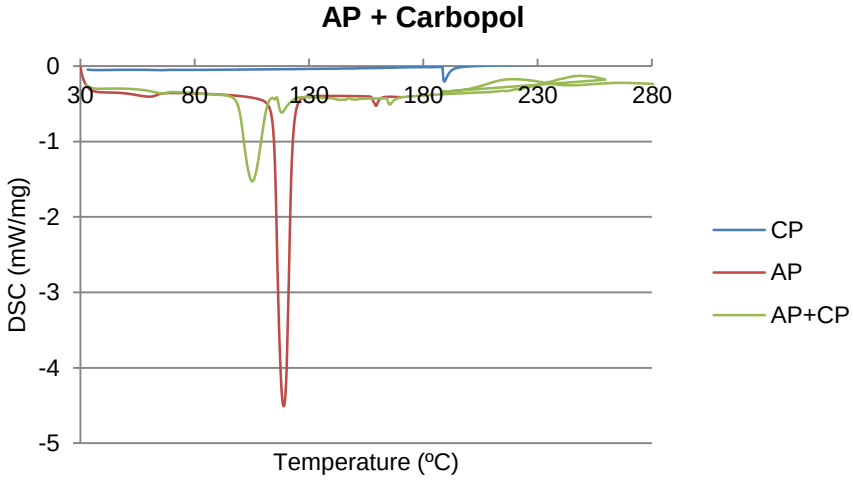


**Figure 58** AP +CP curve of DSC in the heating rate of 10ºC $min^{-1}$

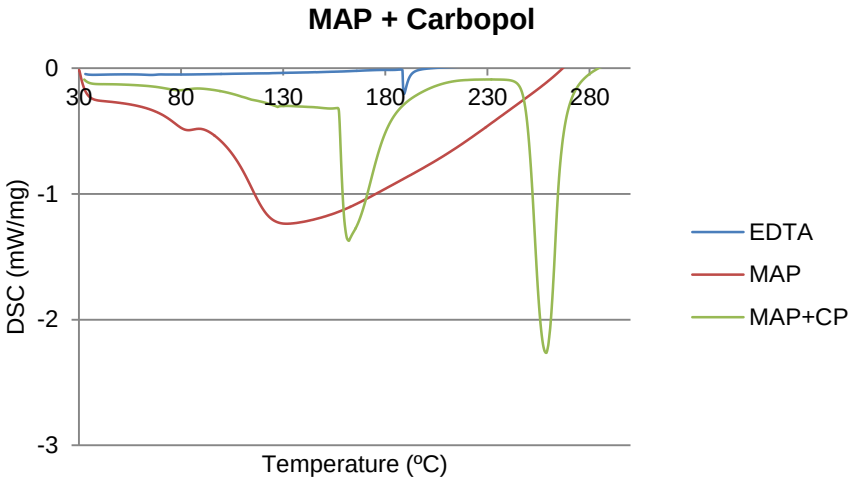


**Figure 59** MAP + CP curve of DSC in the heating rate of 10ºC $min^{-1}$

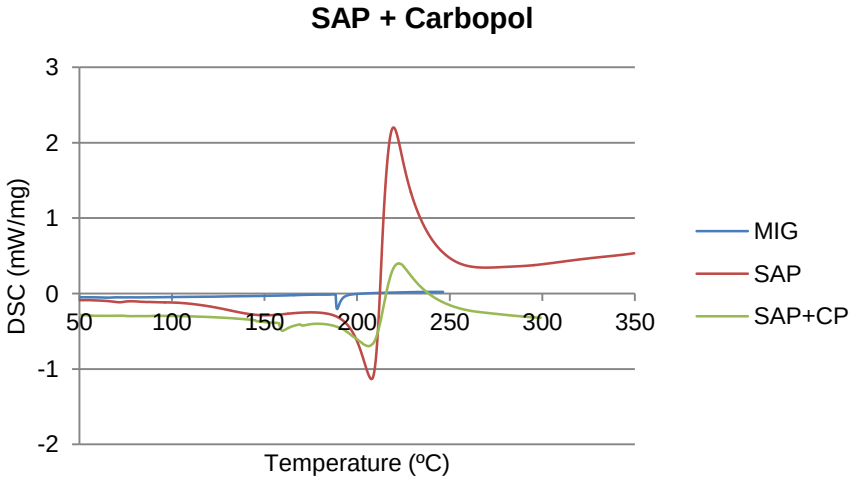


**Figure 60** SAP + CP curve of DSC in the heating rate of 10ºC $min^{-1}$

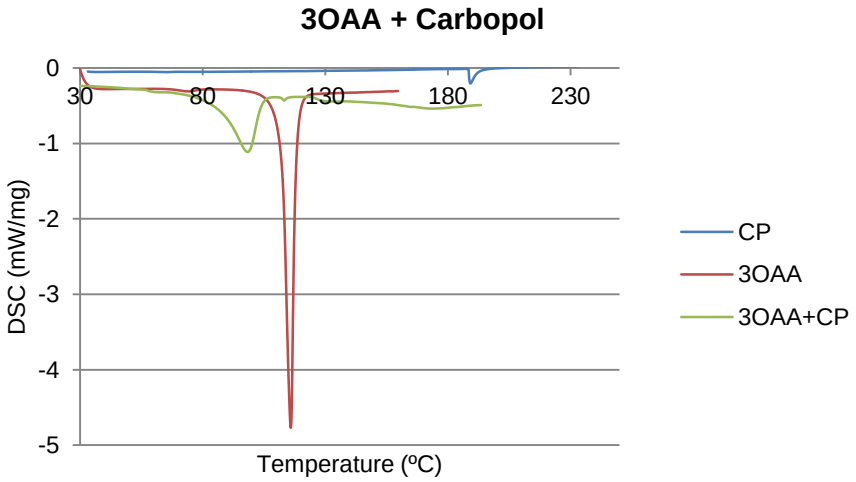


**Figure 61** 3OAA + CP curve of DSC in the heating rate of 10ºC $min^{-1}$

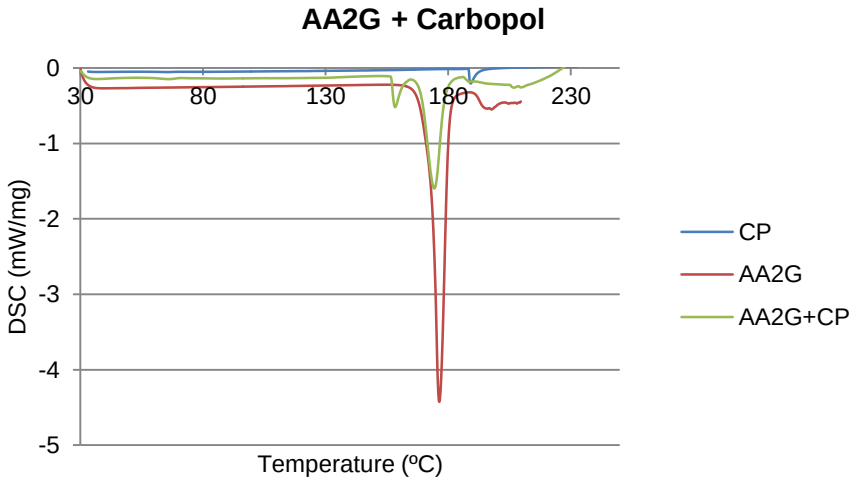


**Figure 62** AA2G + CP curve of DSC in the heating rate of 10ºC $min^{-1}$

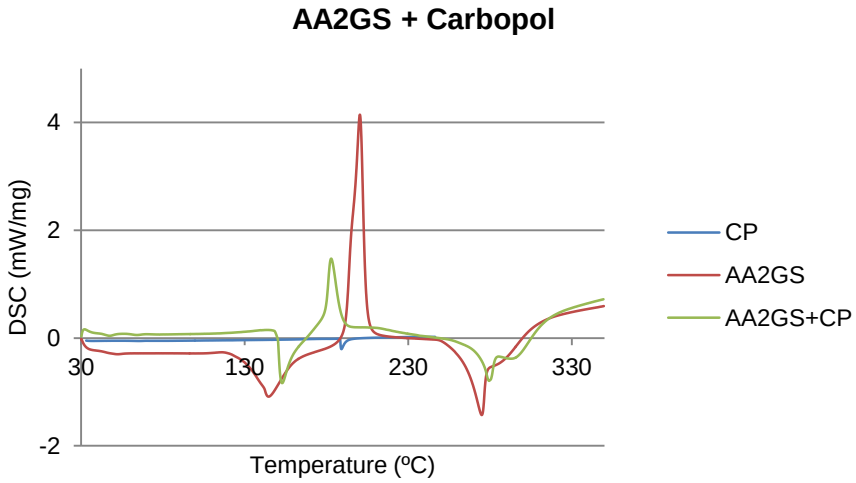


**Figure 63** AA2GS + CP curve of DSC in the heating rate of 10ºC $min^{-1}$

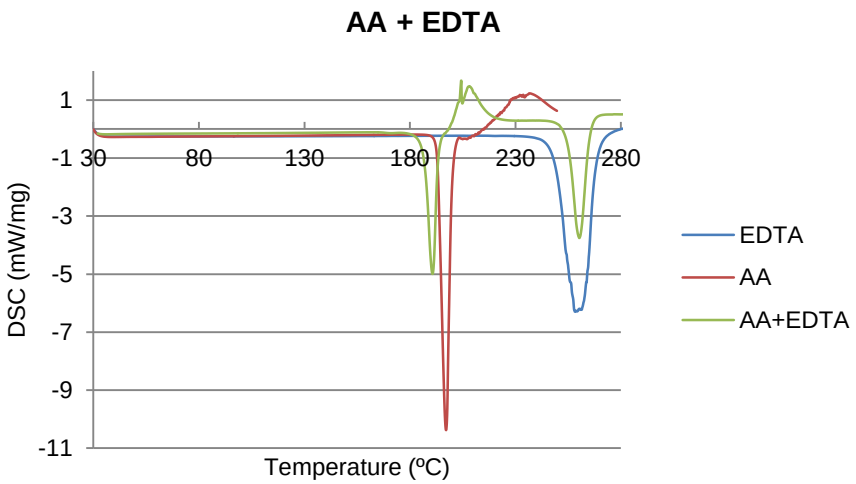


**Figure 64** AA + EDTA curve of DSC in the heating rate of 10ºC $min^{-1}$

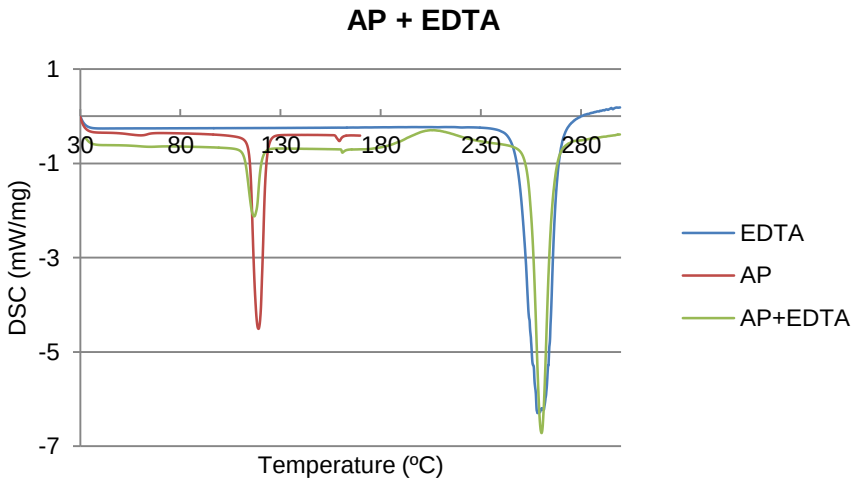


**Figure 65** AP + EDTA curve of DSC in the heating rate of 10ºC $min^{-1}$

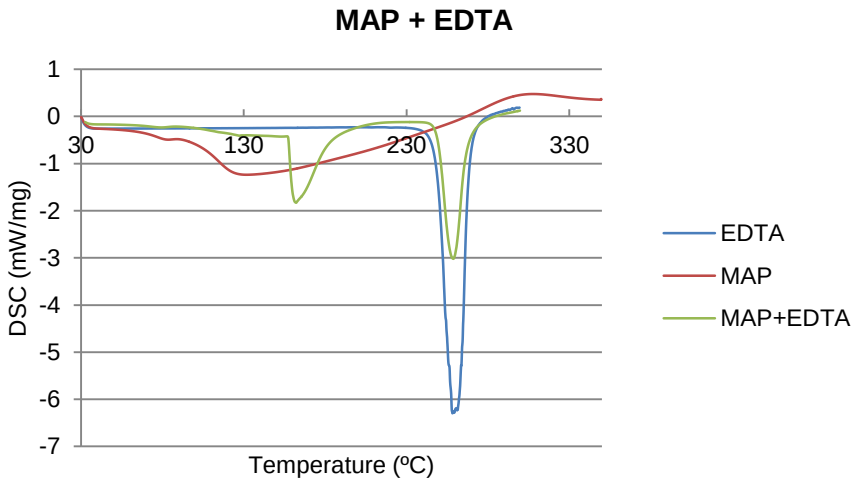


**Figure 66** MAP + EDTA curve of DSC in the heating rate of 10ºC $min^{-1}$

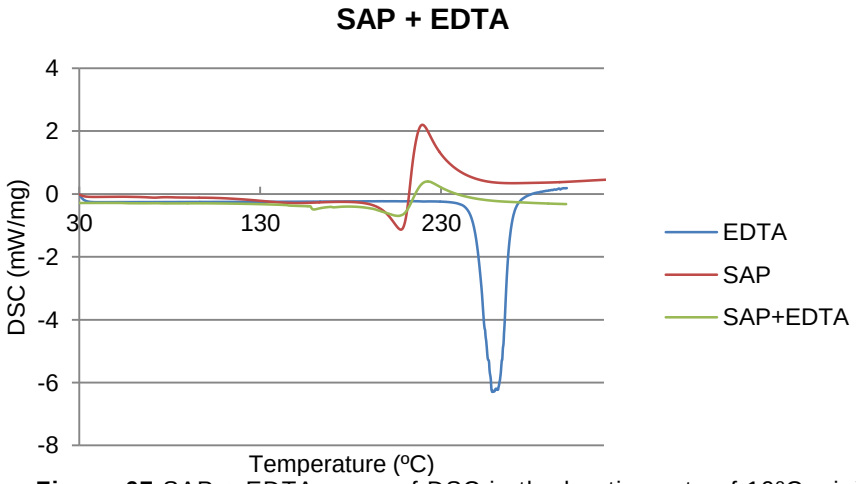


**Figure 67** SAP + EDTA curve of DSC in the heating rate of 10ºC $min^{-1}$

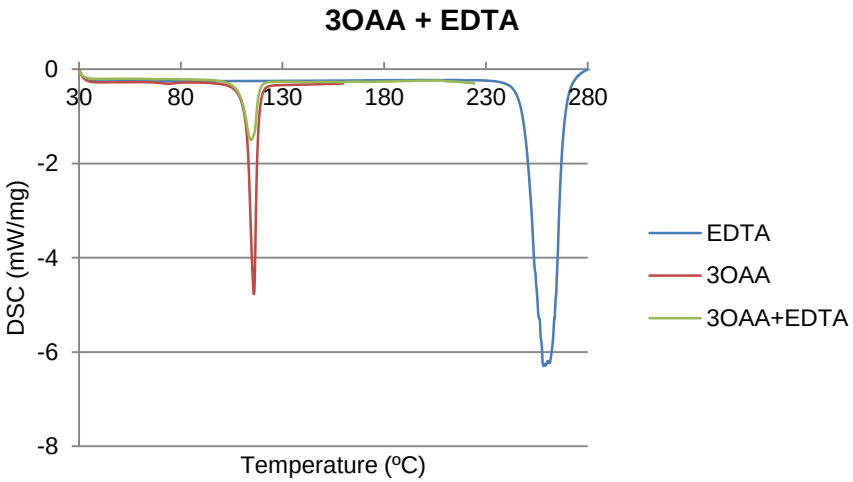


**Figure 68** 3OAA + EDTA curve of DSC in the heating rate of 10ºC $min^{-1}$

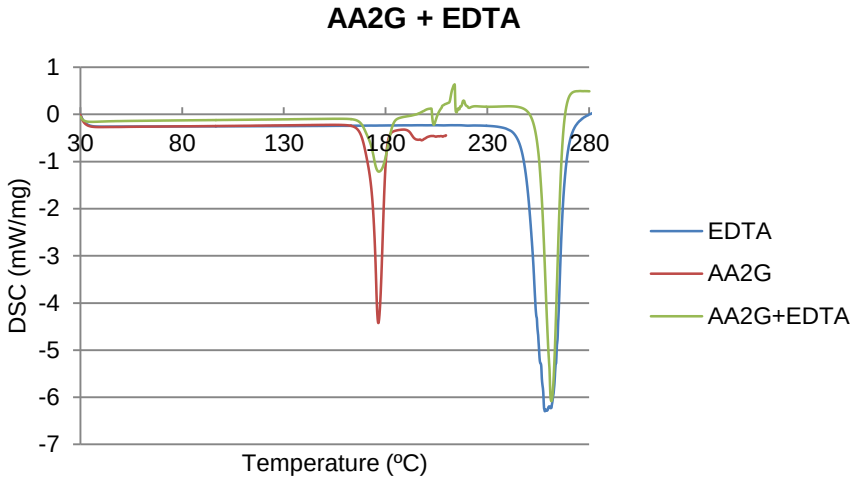


**Figure 69** AA2G + EDTA curve of DSC in the heating rate of 10ºC $min^{-1}$

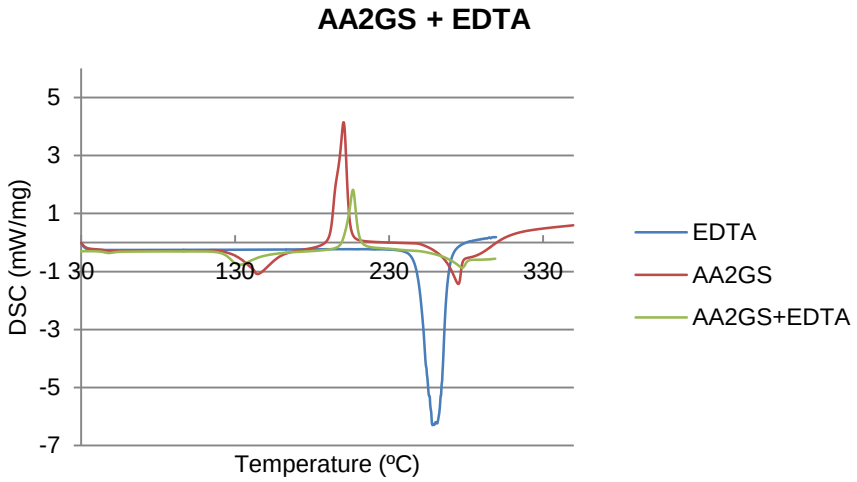


**Figure 70** AA2GS + EDTA curve of DSC in the heating rate of 10ºC $min^{-1}$

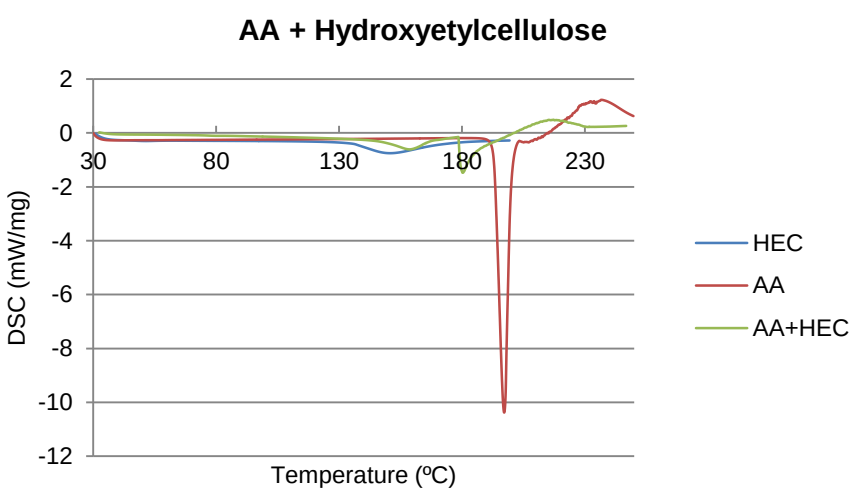


**Figure 71** AA + HEC curve of DSC in the heating rate of 10ºC min$^{-1}$

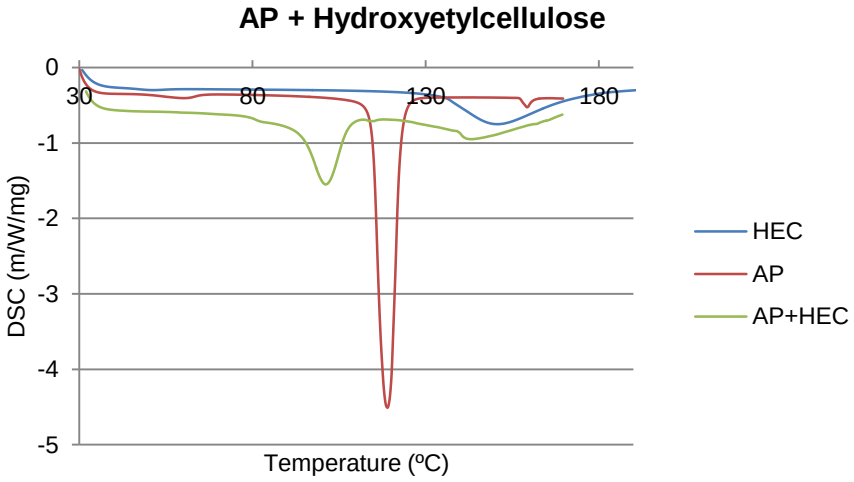


**Figure 72** AP + HEC curve of DSC in the heating rate of 10ºC min$^{-1}$

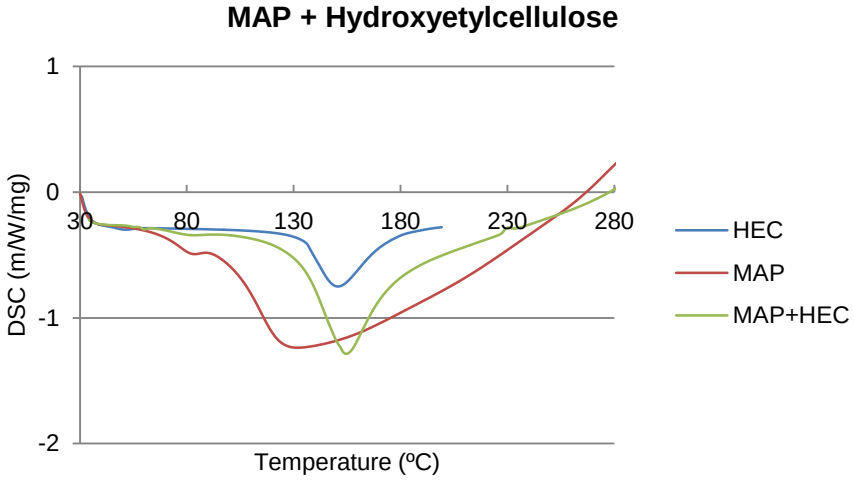


**Figure 73** MAP + EDTA curve of DSC in the heating rate of 10ºC min$^{-1}$

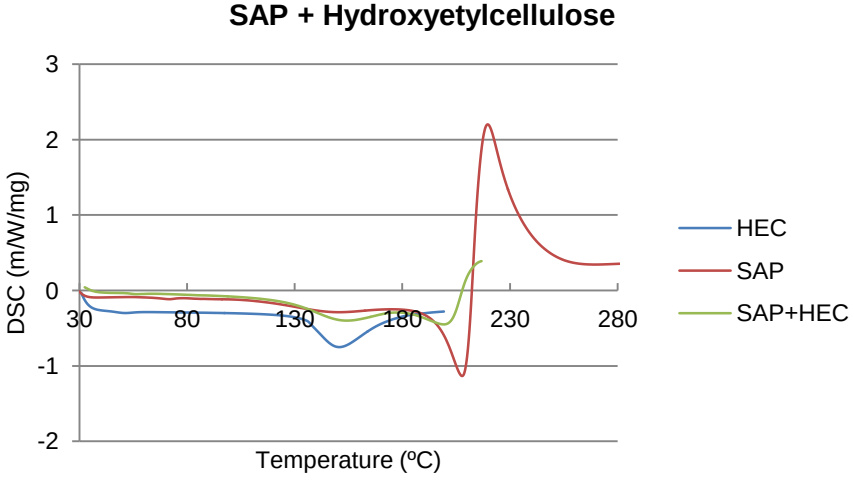


**Figure 74** SAP + EDTA curve of DSC in the heating rate of 10ºC min$^{-1}$

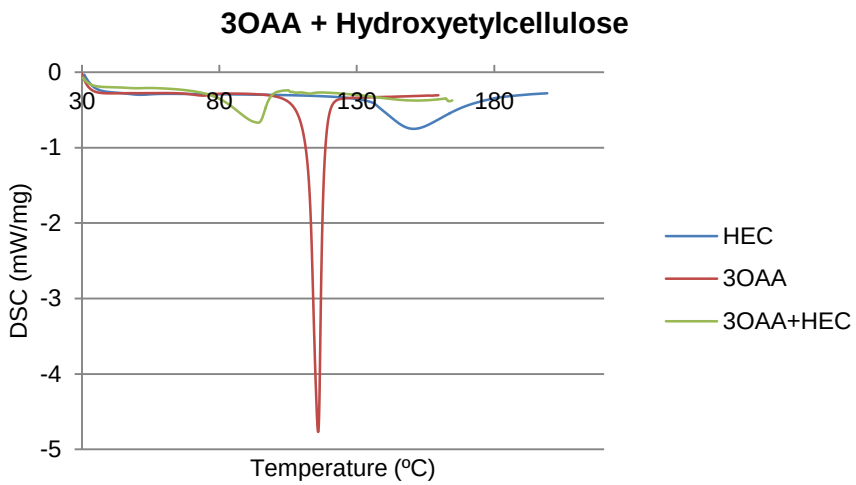


**Figure 75** 3OAA + HEC curve of DSC in the heating rate of 10ºC $min^{-1}$

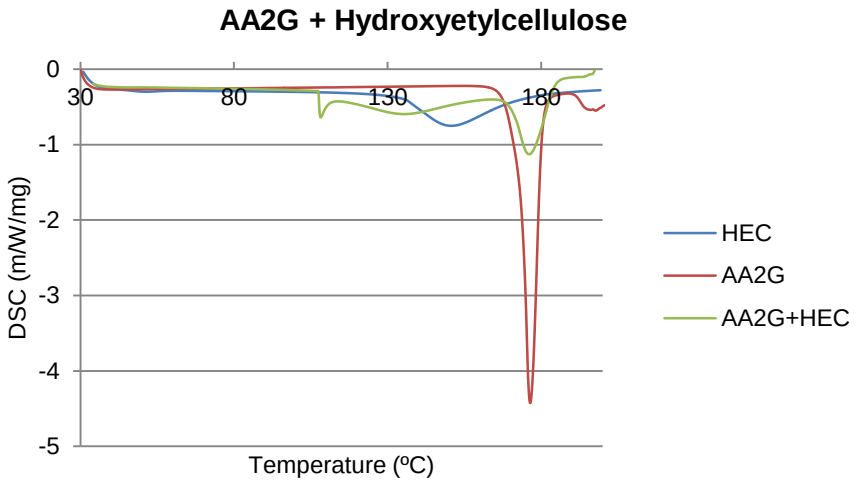


**Figure 76** AA2G + EDTA curve of DSC in the heating rate of 10ºC $min^{-1}$

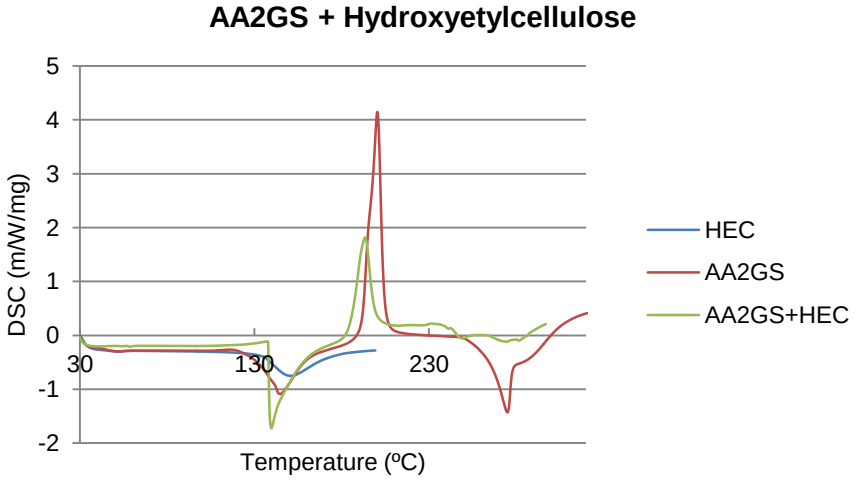


**Figure 77** AA2GS + HEC curve of DSC in the heating rate of 10ºC $min^{-1}$

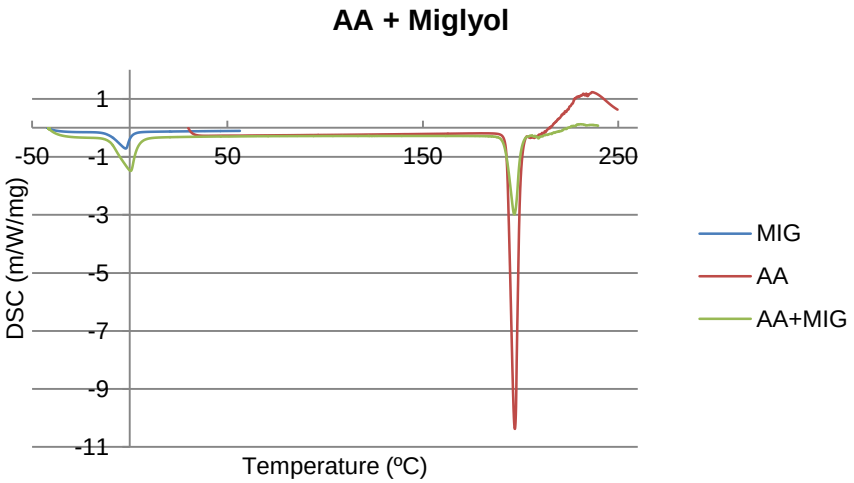


**Figure 78** AA + MIG curve of DSC in the heating rate of 10ºC $min^{-1}$

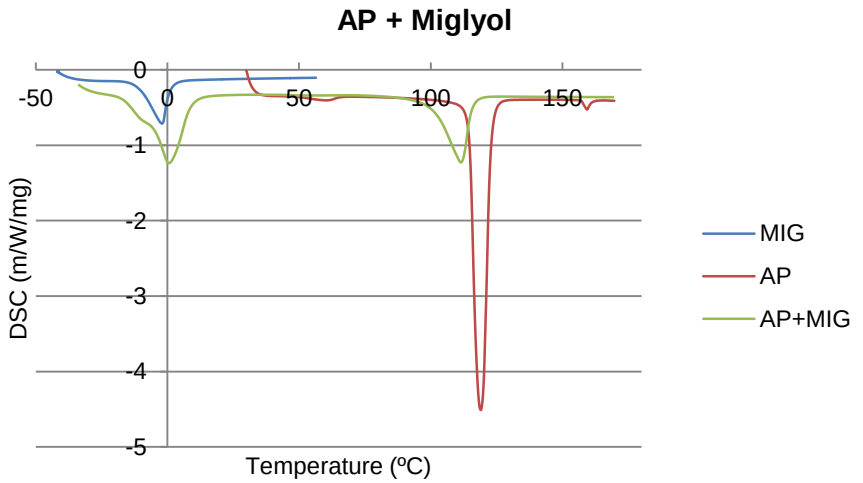


**Figure 79** AP + MIG curve of DSC in the heating rate of 10ºC $min^{-1}$

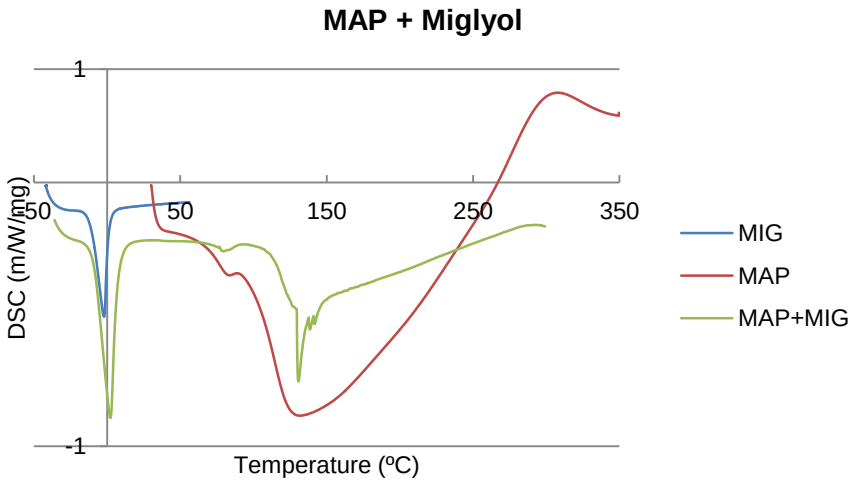


**Figure 80** MAP + MIG curve of DSC in the heating rate of 10ºC $min^{-1}$

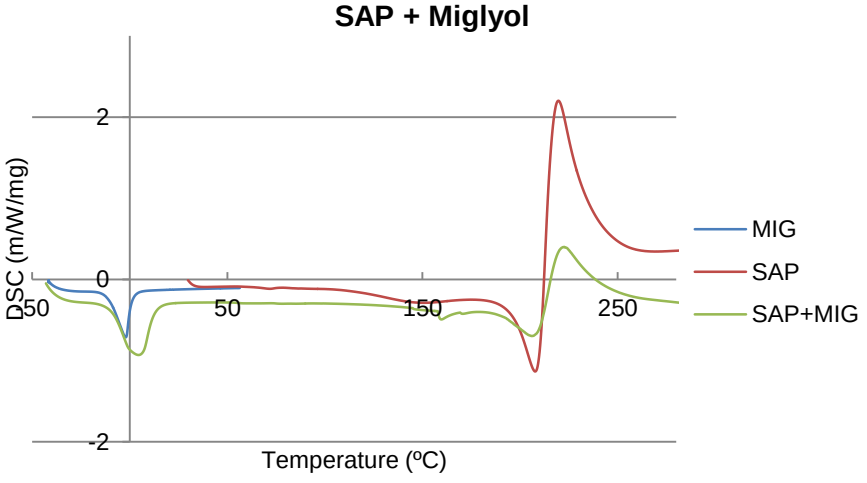


**Figure 81** SAP + MIG curve of DSC in the heating rate of 10ºC $min^{-1}$

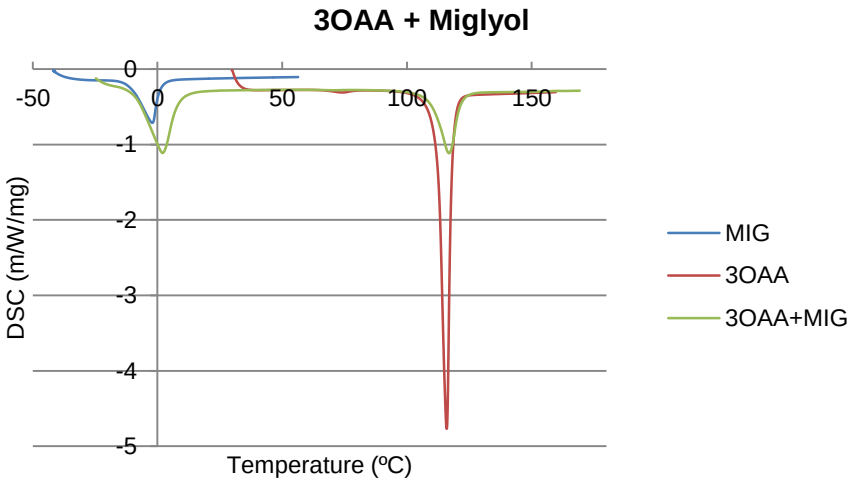


**Figure 82** 3OAA + MIG curve of DSC in the heating rate of 10ºC $min^{-1}$

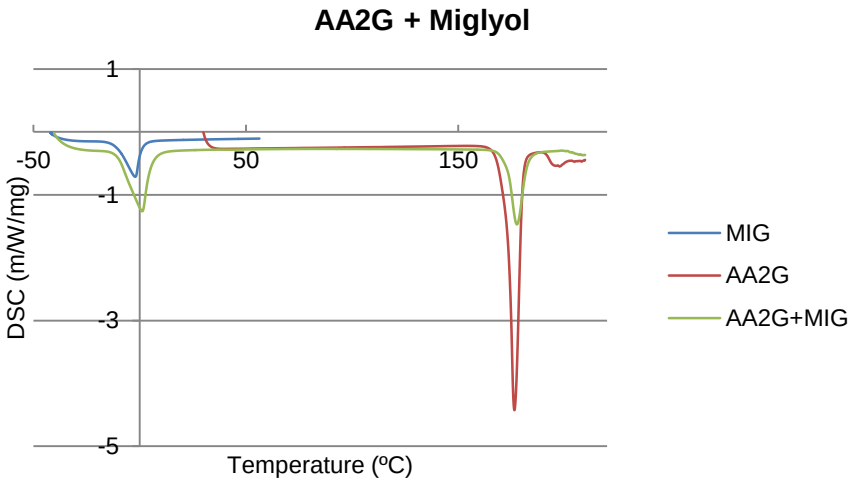


**Figure 83** AA2G + AA2G curve of DSC in the heating rate of 10ºC $min^{-1}$

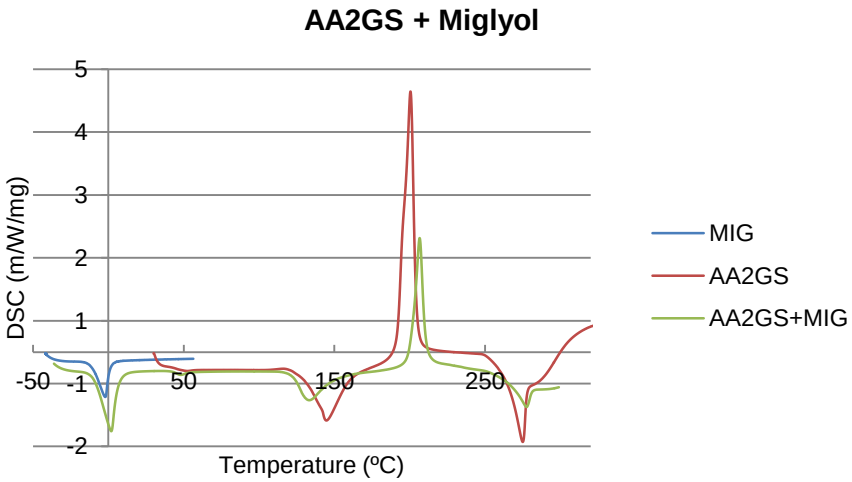


**Figure 84** AA2GS + MIG curve of DSC in the heating rate of 10ºC $min^{-1}$

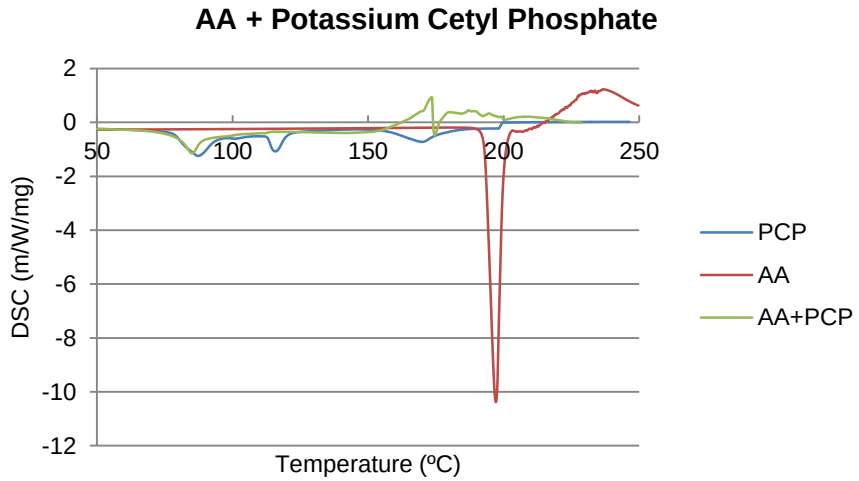


**Figure 335** AA + PCP curve of DSC in the heating rate of 10ºC min$^{-1}$

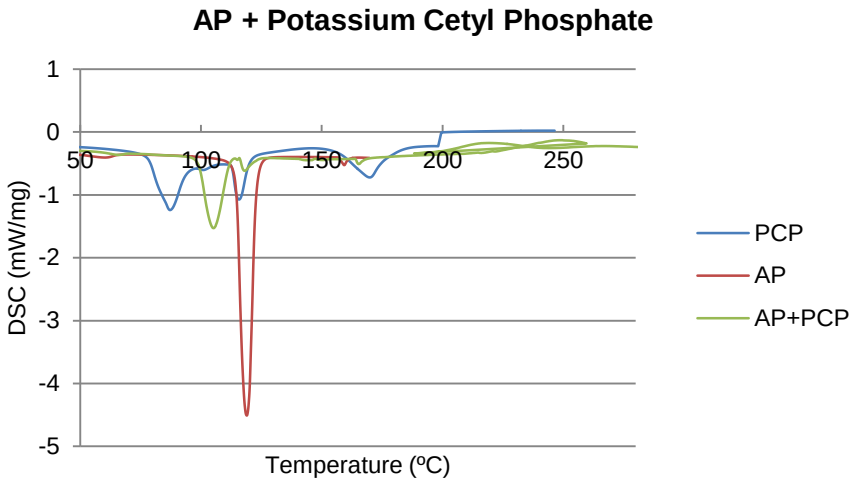


**Figure 32** AP + PCP curve of DSC in the heating rate of 10ºC min$^{-1}$

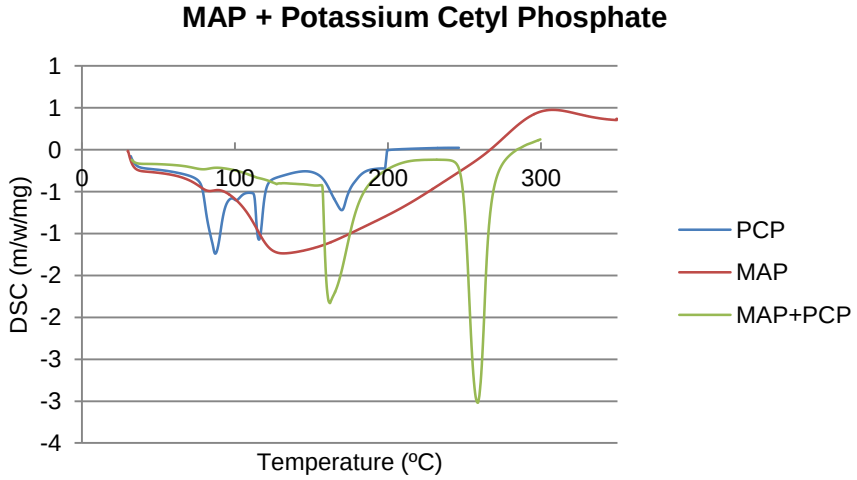


**Figure 87** MAP + PCP curve of DSC in the heating rate of 10ºC min$^{-1}$

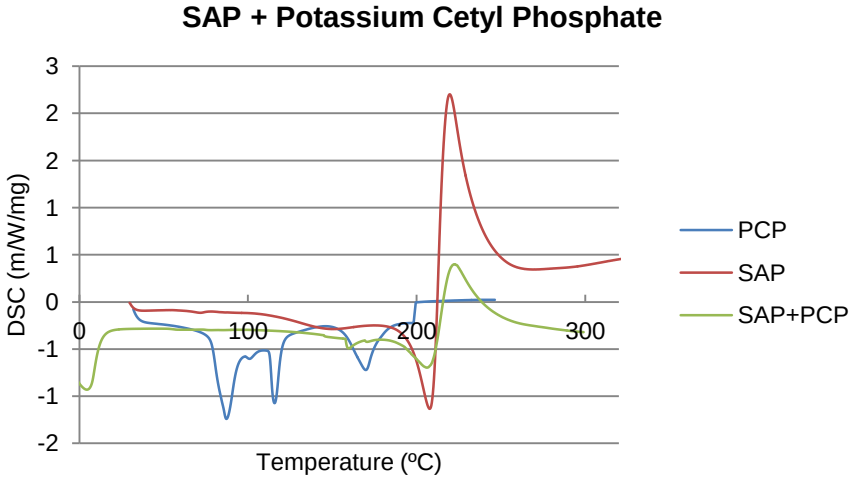


**Figure 88** SAP + PCP curve of DSC in the heating rate of 10ºC min$^{-1}$

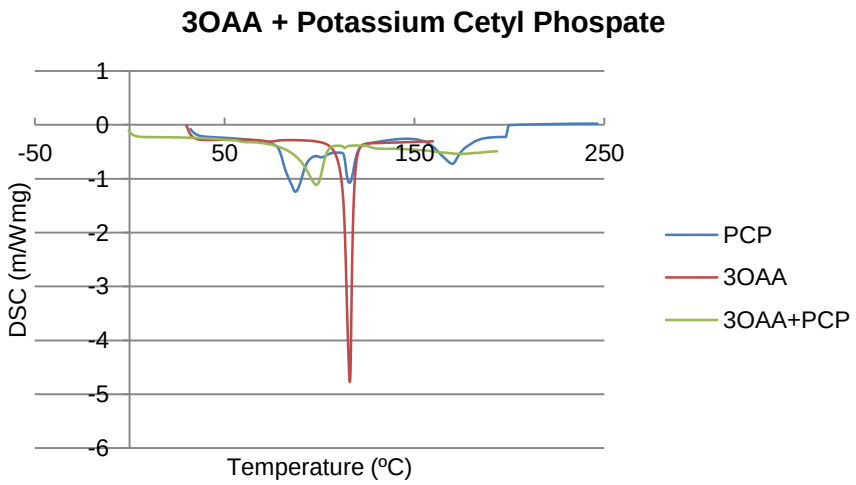


**Figure 89** 3OAA + PCP curve of DSC in the heating rate of 10ºC $min^{-1}$

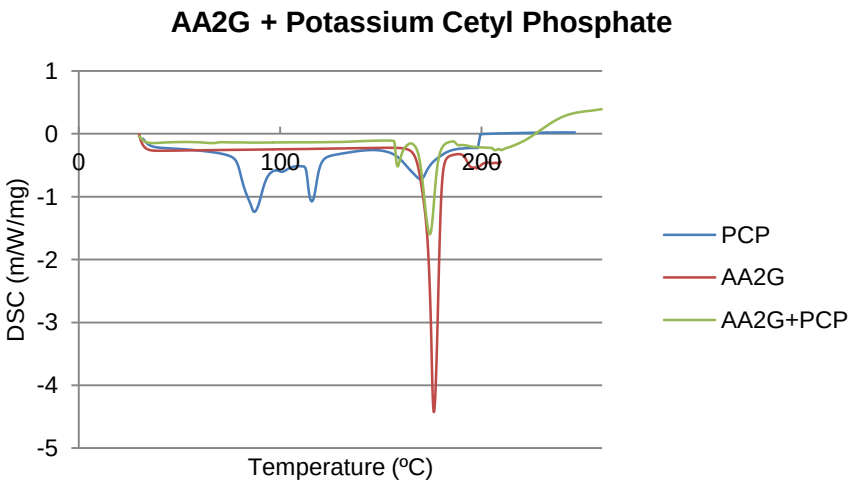


**Figure 90** AA2G + PCP curve of DSC in the heating rate of 10ºC $min^{-1}$

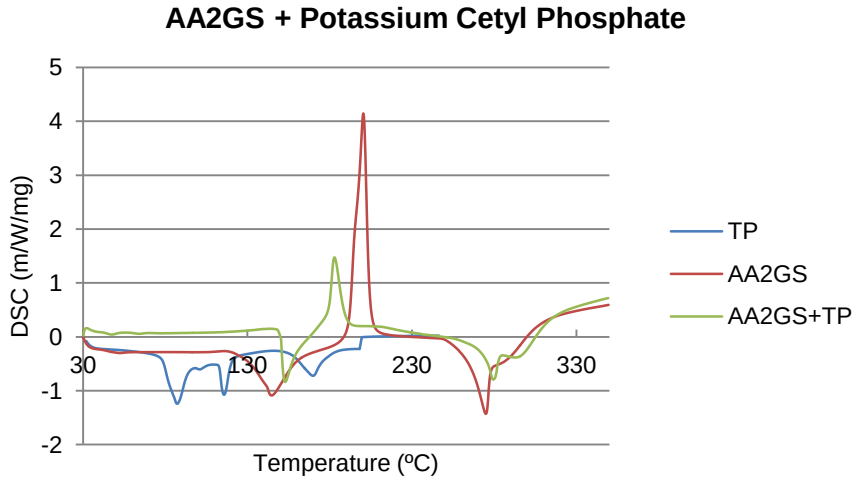


**Figure 34** AA2GS + +CP curve of DSC in the heating rate of 10ºC $min^{-1}$

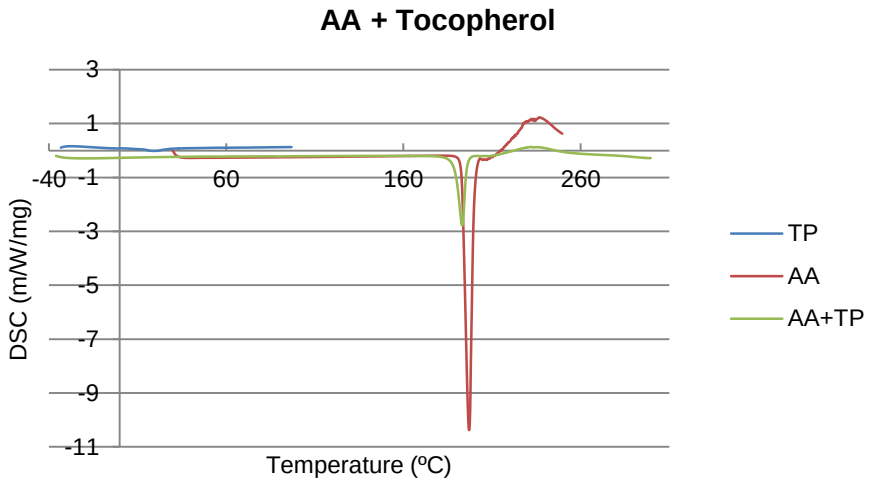


**Figure 92** AA + TP curve of DSC in the heating rate of 10ºC $min^{-1}$

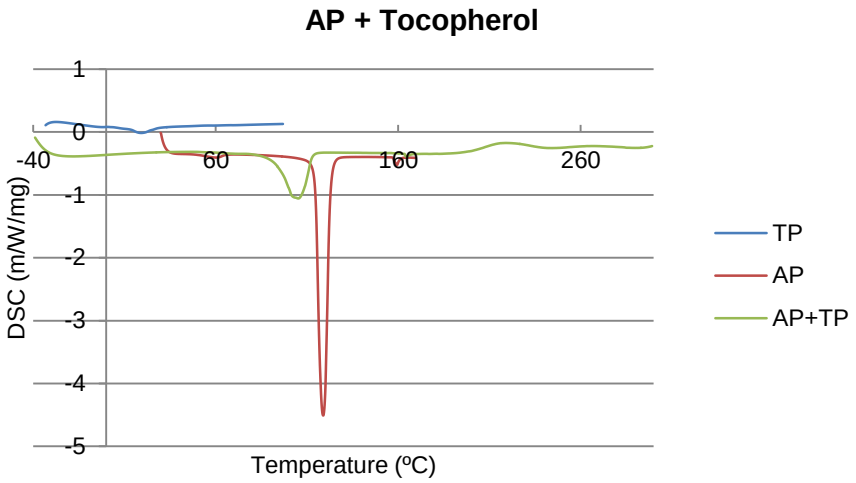


**Figure 93** AP + TP curve of DSC in the heating rate of 10ºC $min^{-1}$

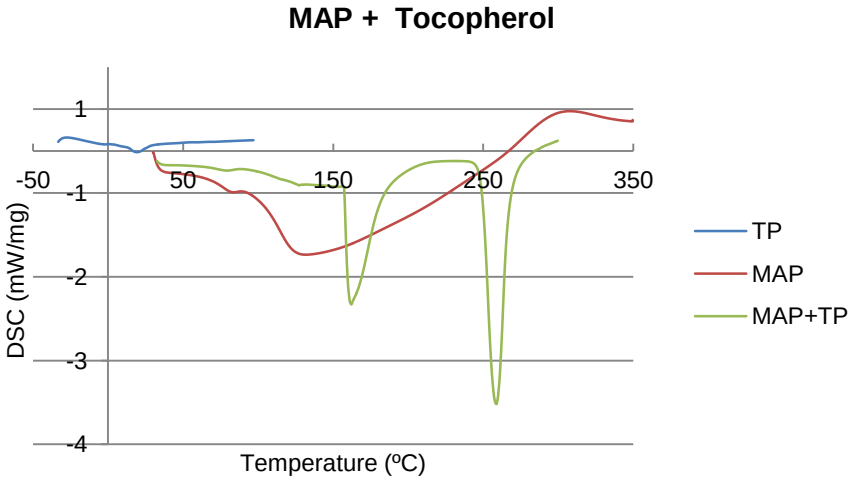


**Figure 94** MAP + TP curve of DSC in the heating rate of 10ºC $min^{-1}$

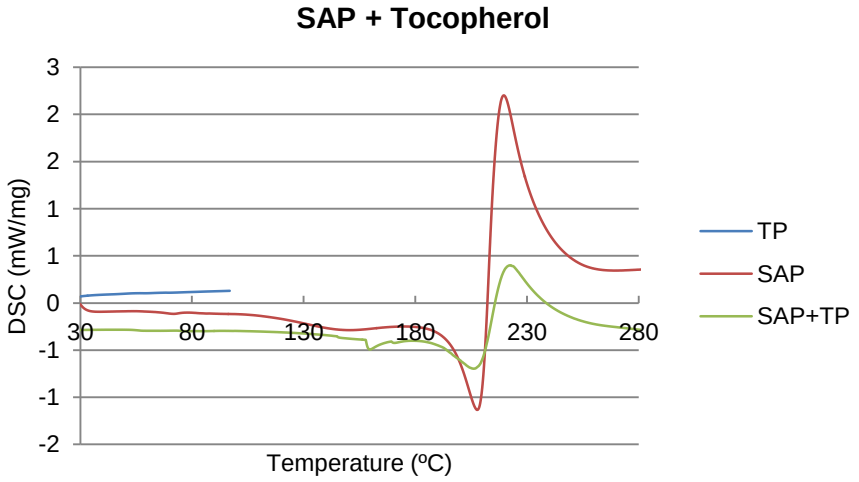


**Figure 95** SAP + TP curve of DSC in the heating rate of 10ºC $min^{-1}$

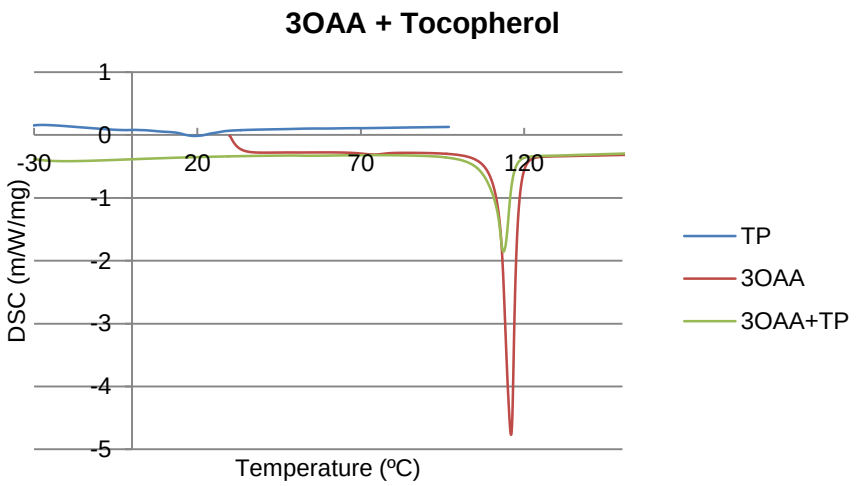


**Figure 96** 3OAA + TP curve of DSC in the heating rate of 10ºC $min^{-1}$

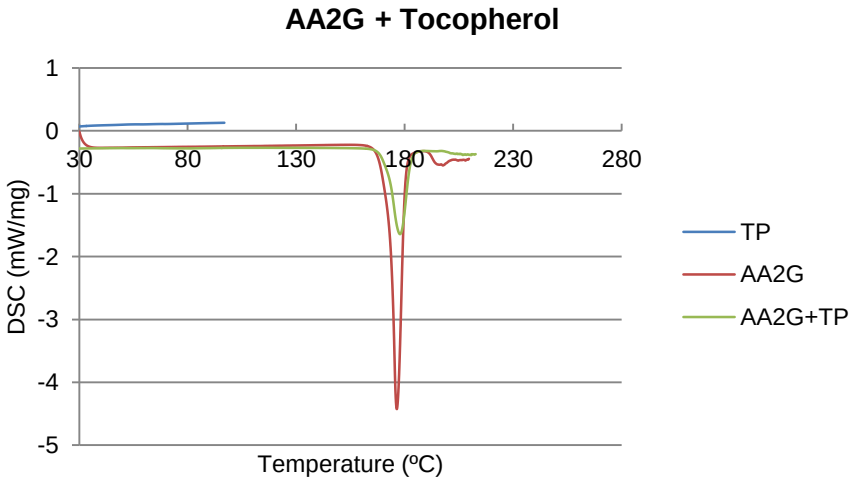


**Figure 97** AA2G + TP curve of DSC in the heating rate of 10ºC $min^{-1}$

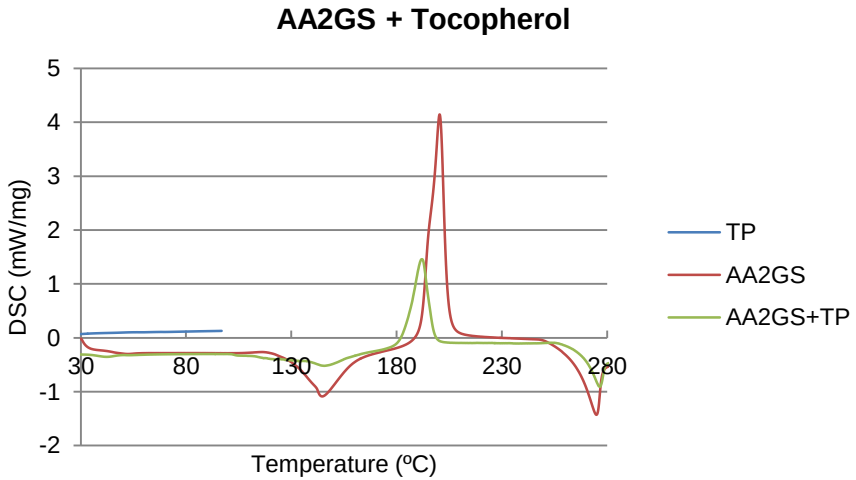


**Figure 98** AA2GS + TP curve of DSC in the heating rate of 10ºC $min^{-1}$

Table 19 summarizes thermoanalytical data of excipients alone and the Table 20 binary mixtures 1:1 (w/w) between AA, AP, MAP, SAP, 3OAA, AA2G and AA2GS plus excipients in the heating rate of 10ºC $min^{-1}$.

**Table 19** DSC thermoanalytical data of excipients in the heating rate of 30ºc $min^{-1}$.

| Isolated excipients | Event | $T_{onset}$/ºC | $T_{peak}$/ºC | ΔH J $g^{-1}$ |
|---|---|---|---|---|
| Stearilic alcohol | Exo | 55.0 | 68.7 | -219.7 |
| Carbopol | Exo | 188.4 | 189.2 | -4.14 |
| EDTA | Exo | 252.3 | 258.2 | -519.6 |
| Hydroxyethylcellulose | Exo | 135.2 | 150.9 | -68.06 |
| Miglyol | Exo | -2.1 | -11.0 | -19.64 |
| Potassium Cetyl Phosphate | Exo | 77.9 | 87.2 | -43.29 |
| | | 112.9 | 115.8 | -18.61 |
| | | 157.4 | 169.9 | -37.92 |
| Tocopherol | Exo | 19.1 | 14.0 | -6.761 |

**Table 20** DSC thermoanalytical data of binary mixtures 1:1 (w/w) between AA, AP, MAP, SAP, 3OAA, AA2G and AA2GS plus excipients in the heating rate of 10ºC $min^{-1}$.

| | Binary mixtures(1:1) (w/w) | $T_{onset}$/ºC | $T_{peak}$/ºC | ΔH J $g^{-1}$ |
|---|---|---|---|---|
| Stearilic alcohol | AA | 190.1 | 194.0 | -155.0 |
| | AP | 80.1 | 83.7 | -60.47 |
| | MAP | 117.7 | 154.2 | -193.3 |
| | SAP | 268.5 | 272.7 | -93.52 |
| | 3OAA | 109.6 | 113.0 | -44.16 |
| | AA2G | 172.8 | 177.1 | -65.79 |
| | AA2GS | 199.5 | 205.3 | 81.44 |
| Carbopol | AA | 196.3 | 238.2 | -52.27 |
| | AP | 115.3 | 118.0 | -4.471 |
| | MAP | 163.1 | 173.8 | -42.44 |
| | SAP | 179.2 | 184.1 | -22.32 |
| | 3OAA | 244.3 | 267.6 | -97.48 |
| | AA2G | 169.1 | 174.4 | -52.41 |
| | AA2GS | 140.0 | 182.9 | 43.34 |
| EDTA | AA | 254.6 | 260.3 | -184.9 |
| | AP | 254.5 | 260.3 | -301.3 |
| | MAP | 248.8 | 258.4 | -245.6 |
| | SAP | 203.14 | 223.12 | -123.44 |
| | 3OAA | 254.4 | 256.6 | -66.01 |
| | AA2G | 253.0 | 261.4 | -296.5 |
| | AA2GS | 213.6 | 227.08 | -195.60 |

| | Binary mixtures(1:1) (w/w) | $T_{onset}$/°C | $T_{peak}$/°C | ΔH J $g^{-1}$ |
|---|---|---|---|---|
| Hydroxyethylcellulose | AA | 178.5 | 180.2 | -88.17 |
| | AP | 94.7 | 101.2 | -54.04 |
| | MAP | 133.2 | 154.6 | -167.8 |
| | SAP | 184.4 | 199.4 | -59.86 |
| | 3OAA | 76.8 | 94.1 | -34.06 |
| | AA2G | 170.3 | 176.1 | -47.37 |
| | AA2GS | 184.9 | 193.4 | 112.3 |
| Miglyol | AA | 191.9 | 196.9 | -96.38 |
| | AP | 101.1 | 111.4 | -49.78 |
| | MAP | 129.4 | 130.7 | -36.83 |
| | SAP | 191.9 | 206 | -71.17 |
| | 3OAA | 110.06 | 119.05 | -34.27 |
| | AA2G | 172.6 | 177.6 | -51.16 |
| | AA2GS | 200.1 | 206.6 | 80.93 |
| Potassium Cetyl Phosphate | AA | 169.0 | 173.4 | -70.12 |
| | AP | 77.2 | 85.1 | -38.75 |
| | MAP | 160.3 | 173.48 | -98.33 |
| | SAP | 190.8 | 206.34 | -43.69 |
| | 3OAA | 173.8 | 174.5 | -15.82 |
| | AA2G | 174.0 | 178.6 | -74.27 |
| | AA2GS | 160.45 | 199.5 | -64.88 |

| | Binary mixtures(1:1) (w/w) | $T_{onset}$/ºC | $T_{peak}$/ºC | ΔH J $g^{-1}$ |
|---|---|---|---|---|
| Tocopherol | AA | 189.0 | 193.2 | -75.87 |
| | AP | 202.3 | 219.3 | -18.12 |
| | MAP | 123.7 | 125.3 | -9.381 |
| | SAP | 167.5 | 201.3 | -53.16 |
| | 3OAA | 205.3 | 209.7 | -15.75 |
| | AA2G | 171.5 | 177.9 | -57.71 |
| | AA2GS | 183.7 | 192.1 | 99.77 |

Another aspect when formulating with antioxidants, is the compatibility with the constituents of the pharmaceutical or cosmetic products. Care must be taken to protect the antioxidants from neutralizing each other when incorporated in the same formulation. Quite often, reactions can occur between the active ingredient and one or more excipients or between active ingredients. In the thermograms of AA, AP, MAP, SAP and AA2G one exothermic peak is observed. In case of AA2GS an endothermic peak is shown. The first one is attributed to the fusion of the all antioxidants which is expected according to the bibliography (Daneluti AL *et al*. 2015; Rowe *et al*. 2009; Roy L, Urooj A, 2013).

With regard to evaluating possible physical-chemical interactions and incompatibilities between antioxidants and excipients, a previous analysis of DSC curves of each pure constituent is necessary. The thermal profile of each excipient under study was traced in the heating rate of 10ºC/min. The results obtained from thermal compatibility study between antioxidants and excipients where DSC curves were overlaid made it possible to measure all the events related to the binary mixtures, as well as some possible interactions between the components. Initially, the AA, AP, MAP, SAP, 3OAA, AA2G and AA2GS thermal behaviour in the calorimetric curve demonstrated a well-defined peak at 197 ºC, 119ºC, 127ºC, 207ºC, 115ºC. 176ºC, 200ºC, respectively. Thus, displacements in the onset temperatures of the melting peak or its disappearance may indicate physical interactions.

After analysing the DSC curves from binary mixtures, it can be assumed that some excipients did not affect some antioxidants thermal behaviour without any apparent physical-chemical change or incompatibility. Among these combinations SAP, AA2GS and

AA22G are the more compatible with the tested excipients: AEE, CP, EDTA, HEC, MIG PCP and TP. Thus, these excipients did not cause significant change in SAP, AA2G and AA2GS thermal behaviour; more specifically, they did not shift its peaks. Only $T_{peak}$ of the AA2G suffer displacement in presence of EDTA and $T_{peak}$ of the SAP shifted in presence of AEE. In respect to AA, AEE, HEC and MIG are excipients that not promote displacement in its $T_{peak}$. However, CP and PCP promotes a slight $T_{peak}$ displacement. EDTA promoted displacement, which can be an indicative of physical interaction.

AP was the antioxidant with more incompatibilities. Only in presence of AEE and MIG were no changes recorded in the $T_{peak}$ of this antioxidant. 3OAA and MAP are other antioxidants with many incompatibilities. 3OAA shows evidence of higher variation in $T_{peak}$ in presence of CP, EDTA, HEC, PCP and TP, and MAP was considerably altered in the presence of AEE, CP, EDTA, HEC and PCP, suggesting in both antioxidants some incompatibility phenomena.

Stearilic alcohol is a solid with melting point between 59.4 and 59.8ºC. The curve of this excipient shows a single endothermic peak which is characteristic of the melting point followed by a mass loss. It can be inferred that this excipient melts earlier than the other tested excipients.

Strong interactions between the antioxidants and EDTA, and PCP can be assumed because the active ingredient melting peak almost disappeared and/or $T_{peak}$ is not possible to be precisely determined. AEE, HEC and MIG are generally compatible excipients, since no alteration of the calorimetric performance of antioxidants was observed in this study.

MIG did not alter any antioxidant $T_{peaks}$, and HEC and AEE in most antioxidants were not found have a negative impact on the antioxidant thermogram. In the case of HEC only interfered at the peak of AP, MAP and 3OAA and in the mixture with AEE only the antioxidants AP, MAP and SAP underwent significant changes.

CP interfered in behaviour of MAP and 3OAA. TP is also an antioxidant and was showed in the results to have been harmoniously compatible with AA and its derivatives (except in case of the AP and 3OAA). The harmoniously compatibility corroborates the widely combination of AA and TP in cosmetics selected in 2015 (Silva 2016). This combination provides very good protection against UVB radiation and has a prophylactic role in defending the skin from photo oxidative damage (Karim A, 2004; Klock J *et al.* 2005; Godic A *et al*. 2014; Lavecchia R, Zuorro A, 2015; Lobo V *et al.* 2010).

AA, in addition to its role in skin, plays a role in the stabilization of TP in the formulations; thus, AA maintains the active form of TP. Pandel and Poljsak attribute to this combination the antioxidant synergic potential between TP and AA. They demonstrate in their work that AA can regenerate α-tocopherol from its chromanoxyl radical. Pouillot *et al*. 2011, suggested that the best current approach is to combine antioxidants acting in synergy. This means that, the global effects of these combined antioxidants will be greater than the sum of each one's specific effects.

However, these incompatibilities need to be further investigated by another analytical technique as the Fourrier transformed infrared spectroscopy (FTIR) is to confirm and exclude this phenomenon (Almeida M *et al*. 2010; Daneluti AL *et al*. 2015).

In majority of the cases, melting point of the antioxidant was well preserved with slight changes in terms of broadening or shifting toward a lower temperature. It has been reported that the quantity of material used, especially in active-excipient mixtures, affects the peak shape and enthalpy (Bae HK *et al*. 2002; Baertschi S, 2006; Bezerra G *et al*. 2016).

Thus the minor changes in the melting temperature of active ingredient can be attributed to the mixing of active and excipient, which lowers the purity of each component in the mixture and may not necessarily indicate potential incompatibility (Bae HK *et al*. 2002; Almeida M *et al*. 2010; Daneluti AL *et al*. 2015; Bezerra G *et al*. 2016).

Variations in the enthalpy values for the binary mixtures can be attributed to some heterogeny in the small samples used in experiments. The enthalpy values are reduced to half in the binary mixtures mentioned. It is due to the fact that in the mixtures the mass is 50% of excipient and 50% of antioxidant (Almeida M *et al*. 2010; Daneluti AL *et al*. 2015).

### *IV.3.5. Antioxidant activity*

Results were expressed as the concentration (in µM) of antioxidant that causes 50% reduction in the DPPH colour ($EC_{50}$ Table 21) (Oroian M *et al.* 2015).

**Table 21** Antioxidant activity of each antioxidant given by $EC_{50}$ values (µM).

| Antioxidant | $EC_{50}$ | SD± |
|---|---|---|
| AA | 1.54 | 0.213 |
| AP | 32.2 | 0.355 |
| MAP | 45.2 | 0.424 |
| SAP | 5.00 | 0.340 |
| 3OAA | 4.12 | 0.345 |
| AA2G | 9.15 | 0.134 |
| AA2GS | 5.28 | 0.360 |
| Rutin | 4.68 | 0.50499 |

The results showed that all the seven antioxidants were able to reduce DPPH at the tested concentrations. Among the tested compounds, rutin and AA exhibited the highest radical scavenging activity within the range described by other authors (Smaoui S *et al.* 2013; Smart RC, Crawford CL, 1991; Snape T *et al.* 2010).

AA is the antioxidant with highest scavenging activity translated by a lower $EC_{50}$ value. Derivatives like SAP, 3OAA, AA2GS and AA2G showed similar scavenging activity. AA was found to be around two times more efficient than 3OAA, SAP and AA2GS.

Based on these observations, the following order of the radical scavenging activity of the studied antioxidants is proposed: AA > 3OAA > SAP > AA2GS > AA2G > AP > MAP. The resulting order can be explained by the structure of derivatives. Studies of the relationship between the chemical structure and their capacity to scavenge free radicals have shown that the scavenging activity depends on the number, position and nature of the substitutes of the AA ring. These parameters are also linked to the polarity of the components (Assimopoulou AN *et al*. 2005; Graf E, 1992; Hacisekvi A, 2009; Takebayashi J *et al.* 2008; Takebayashi J *et al.* 2006; Takebayashi J *et al*. 2002).

Ascorbyl phosphate salts being prodrugs must be first converted by an enzymatic hydrolytic process before penetrating into the skin to exerts their antioxidant activity like AA. It has been showed that even though AA2G has a higher stability its antioxidant capacity was lower than the activity in vivo of ascorbic acid and ascorbyl palmitate. This can be explained by poor penetration of this compound through human stratum corneum due to its high hydrophilicity (Burke KE, 2004; Cadenas E, Packer L, 2007; Nikki T *et al.* 2016).

By its turn, the 3OAA masks the 3-OH, preventing its ionization and the subsequent oxidation of AA. Although the 3-O-ethyl derivative is also predicted to be more lipophilic than AA because of the added lipophilic ethyl group and hence exhibit improved permeation of the skin. It is unlikely to deliver AA because ethers such as 3OAA can only undergo reversion to AA through a CYP-mediated alpha oxidation of the ethyl group to give an alpha-hydroxyethyl hemiacetal derivative, which can then spontaneously decompose to an aldehyde (acetaldehyde in this case) and AA (Nikki T *et al.* 2016).

Based in previous reasons, is remarkable that in AA structure both the 3-OH and the 2-OH groups are essential for its antioxidant properties. So, those derivatives express minor antioxidant activity than AA.

# Chapter V: Conclusion

## Chapter V: Conclusions

Inserted in the context of an increase in the average life expectancy of the population, there is a greater concern with aesthetics, and higher expectations of consumers regarding the efficacy of anti-aging cosmetics. Faced with this insistent demand, the dermocosmetic companies have to seek new cosmetologically active principles. Antioxidants were highlighted in this work as active ingredients of anti-aging cosmetics. The structural differences between AA and its derivatives and the lack of comparative studies in the literature motivated the comparison between them, aiming to obtain insight regarding their use in cosmetic formulations. The study also contemplates a new derivative of AA: sulphate ascorbic acid-2-glucoside.

A large number of topical preparations are available in the market, containing AA and its derivatives. The formulation of these preparations can be troublesome due to the instability of AA. This fact fostered the development of new derivatives able to overcome the several physical and chemical factors related to the instability of AA. These parameters have been highlighted in this work by determining the solubility in water and glycerol, the stability in different contexts of pH, temperature, presence of metals and UV light, that were investigated for AA and its derivatives: AP, MAP, SAP, 3OAA, AA2G and AA2GS.Their antioxidant activities were also investigated and compatibilities with frequently used excipients in topical formulations were studied.

In a first analysis it was possible to conclude that 3OAA and AA2G solubility in water was higher than 1000 mg/ml at room temperature, and, in turn, AA, MAP, SAP are classified as “freely soluble” and AP are remarkably the unique “very slightly soluble of the antioxidants in study. AA and its derivatives are, ones more than others, unstable molecules. Although these antioxidants are soluble in water, in this medium have high propensity to oxidise and/or degrade due to attack by water elements and for this reason could be interesting to use glycerol as solvent. These antioxidants did not demonstrate higher solubility in glycerol in comparison with water. The most soluble derivative in water was 3OAA and in glycerol was SAP.

Considering the pH stability results, in the pH range of 4 to 5 the decomposition is controlled, while at pH 7 to 8 it increases. For SAP and MAP salts, neutral or basic solutions guarantee the highest stability, whereas AP was found to be stable at acidic pH similar to AA. The 3OAA antioxidant presented the highest stability at pH 5 along the study period. AA2G and AA2GS are the derivatives that are more stable in broadly pH

values. As mild acidic conditions rather than basic conditions are suitable for the formulation of topical preparations due to the compatibility with skin pH, the 3OAA, AA2G and AA2GS and even AP are interesting antioxidants to be included in skin products.

Relatively to thermal stability, data demonstrate that concentration of AA is more constant at room temperature followed by refrigerated conditions. SAP is more stable than MAP in the long-term studies for all temperatures. AA2G exhibited the higher stability at room temperature while AA2GS at 4ºC. 3OAA showed higher stability at 4ºC and 25ºC.

In which concerns the stability in the presence of metallic ions, the addition of Fe(II), Fe(III), Cu(II) and Ca(II) ions to AA decreases its stability in opposite to Mg(II). The AP was the most unstable and MAP, SAP and 3OAA exhibited moderate stability in the presence of $Fe^{2+}$ and $Fe^{3+}$ ions. AA2G and AA2GS showed much higher stability against $Ca^{2+}$, $Cu^{2+}$ and $Mg^{2+}$ than AA.

AA and AP are the antioxidants that demonstrate more instability in presence of UV light. The other antioxidants demonstrate good stability in all periods of testing.

Overall, concerning their stability, AA was found to be the most unstable and 3OAA and AA2G the most stable compounds. The new derivative showed favourable properties when compared to other AA derivatives like AP.

With respect to thermal analysis of AA, AP, MAP, SAP and AA2G one exothermic peak was detected. In case of AA2GS an endothermic peak was observed. After analysing the thermograms from binary mixtures among these combinations SAP, AA2GS and AA22G are the more compatible with the tested excipients: AEE, CP, EDTA, HEC, MIG PCP and TP. Only $T_{peak}$ of the AA2G suffer displacement in presence of EDTA and $T_{peak}$ of the SAP is shifted in presence of AEE. In respect to AA, AEE, HEC and MIG are excipients that not promote displacement in its $T_{peak}$ However, CP and PCP promotes a slight $T_{peak}$ displacement. EDTA promoted displacement, which can be an indicative of physical interaction. AP was the antioxidant with more incompatibilities. Only in presence of AEE and MIG were no changes recorded in the $T_{peak}$ of this antioxidant. 3OAA and MAP are other antioxidants with many incompatibilities. 3OAA shows evidence of higher variation in $T_{peak}$ in presence of CP, EDTA, HEC, PCP and TP, and MAP was considerably altered in the presence of AEE, CP, EDTA, HEC and PCP, suggesting in both antioxidants some incompatibility phenomena.

The following order of the radical scavenging activity of the studied antioxidants was found: AA > 3OAA > SAP > AA2GS > AA2G > AP > MAP. Therefore, a new derivative of AA was found to have antioxidant properties and a better stability profile than AA.

Moreover, this study puts in evidence 3OAA and AA2G as the best AA derivatives in which concerns physical chemical properties. AA2GS is an antioxidant with reasonable stability in solution even at different temperatures, pH, and in presence of metal ions, optimal compatibility and high antioxidant capacity. These factors may encourage its inclusion in anti-aging cosmetics by these facts that help maintaining the final product quality.

To achieve the stabilization of AA in semi-solids it is necessary controlling the pH and electrolyte concentration. Along with other factors, formulation type also plays an important role in the stability of AA and its derivatives. The formulator has to adopt appropriate measures to achieve the stabilization of the drug during its storage period (preferable in cool or room temperature) and use.

For the formulation of oxidizable and ionizable substances such as ascorbic acid or derivatives in an emulsion or cream preparation, factors such as solubility, pH, viscosity, polarity of the medium, choice of emulsion type and emulsifying agent, hydrophilic-lipophilic balance (HLB) value of the system, compatibility of the active with formulation ingredients, use of appropriate antioxidant/preservative, etc. should be considered.

To overcome some aspects and facilitate the production of cosmetics with anti-aging antioxidants based on AA is preferable to select a derivative like 3OAA, AA2G or AA2GS or SAP and MAP. The results confirmed the capability of these compounds to protect vitamin from degradation. Thus confirming that derivatives as stable derivatives of vitamin C that may be easily used in cosmetic products.

# Chapter VI: Bibliography

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