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2.º CICLO

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Monitoring the nutritional quality of tomatoes during shelf life after a post-harvest treatment with calcium chloride

Ana Isabel Koch de Oliveira

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Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
Biologia Funcional e Biotecnologia de Plantas

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Ana Isabel Koch de Oliveira
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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Resumo

O tomate (*Solanum lycopersicum* L.) é um alimento consumido à escala mundial, fresco ou processado, sendo altamente apreciado pelas suas características organoléticas e pelo seu valor nutricional. Por estas razões, a produção de tomate tem aumentado bastante nos últimos cinquenta anos, sendo considerada a categoria dos vegetais mais importante do ponto de vista económico. O consumo de tomate tem sido associado a um reduzido risco de doenças, de que são exemplo doenças cardiovasculares e cancro. Estes benefícios para a saúde são atribuídos a vários compostos com atividade antioxidante que estão presentes no tomate, em particular a carotenoides e compostos fenólicos. Uma grande percentagem da produção de frutos e vegetais é perdida devido a falhas na preservação e manuseio dos alimentos. Para evitar estas perdas pós-colheita e a consequente perda de qualidade nutricional, têm sido aplicados vários tratamentos a culturas hortícolas frescas. O cloreto de cálcio ($CaCl_2$), uma das várias substâncias usadas nestes tratamentos, é considerado responsável por diminuir a deterioração e aumentar o tempo de vida de prateleira de frutos e vegetais. No entanto, o impacto do $CaCl_2$ no valor nutricional dos tomates não está totalmente descrito, e estudos anteriores recomendam o uso de diferentes concentrações de $CaCl_2$ como as ideais para aumentar o tempo de vida de prateleira destes frutos. Com este trabalho pretende-se avaliar a influência de tratamentos pós-colheita com diferentes concentrações de $CaCl_2$ em vários parâmetros de qualidade de tomates durante duas semanas de armazenamento. O conteúdo total de fenóis e a capacidade antiradicalar do DPPH de extratos metanólicos obtidos de frutos tratados foram avaliados após duas semanas de armazenamento. Os principais compostos fenólicos foram identificados por HPLC-MS com vista a avaliar o impacto do tratamento com $CaCl_2$ no perfil fenólico dos tomates. A metodologia de DPPH-online foi usada para identificar os principais compostos fenólicos que contribuem para a atividade antioxidante dos frutos. A glutationa, um tripéptido com um papel central no sistema antioxidante e indicador do estado redox dos frutos, foi também quantificada por HPLC-MS. A atividade de enzimas envolvidas na síntese (γ-glutamil-cisteinil sintetase e glutamina sintetase) e reciclagem (glutationa redutase) foram avaliadas em extratos proteicos de tomates tratados. Os resultados obtidos sugerem que os tratamentos com 1% e 2% de $CaCl_2$ (p/v) não tiveram um impacto significativo nas características morfológicas dos tomates. No entanto, o tratamento com 6% de $CaCl_2$ (p/v) favoreceu o

desenvolvimento de micro-organismos. Apesar de o tratamento com 2% de $CaCl_2$ (p/v) ter aumentado a concentração de alguns compostos fenólicos (em particular do ácido tricafeoilquínico), o conteúdo total de compostos fenólicos e a atividade antioxidante dos tomates não foi significativamente afetada pelo tratamento. O cloreto de cálcio afetou outros componentes do sistema antioxidante além dos compostos fenólicos. O impacto dos tratamentos com $CaCl_2$ nas enzimas relacionadas com o metabolismo da glutationa resultou num rácio glutationa reduzida/oxidada mais baixo, diminuindo o estado redox dos tomates tratados. Conclui-se ainda que o $CaCl_2$ preveniu a degradação das proteínas com o decorrer do tempo, uma vez que os frutos tratados tiveram um conteúdo proteico significativamente mais elevado do que os controlos.

Abstract

Tomato (*Solanum lycopersicum* L.) is consumed worldwide, fresh or processed, and it is highly appreciated for its organoleptic properties and nutritional value. For these reasons, tomato production has been greatly increasing in the last fifty years and is considered the most important economically in the vegetable category. Consumption of tomato has been associated with reduced risk of several diseases such as cardiovascular disease and cancer. These health benefits are attributed to several compounds with antioxidant activity present in tomato, in particular carotenoids and phenolic compounds. A great percentage of fruits and vegetables production is loss due to unsatisfactory preservation and handling processes. In order to avoid post-harvest damage and decrease of nutritional quality, several treatments have been applied to fresh horticultural crops. Calcium chloride ($CaCl_2$), one of the numerous substances used in these treatments, is reported to reduce deterioration and increase shelf life of fruits and vegetables. However, $CaCl_2$ impact on tomatoes nutritional value is not fully described, and previous studies have recommended the use of different $CaCl_2$ concentrations as the ideal ones for increasing tomato fruits shelf life. This work aimed at evaluating the influence of post-harvest treatments with different $CaCl_2$ concentrations in several quality parameters of tomato fruits during two weeks of storage. The total phenolic content and DPPH scavenging capacity of methanolic extracts obtained from treated tomato fruits were evaluated at the end of storage. The main phenolic compounds were also identified by HPLC-MS in order to evaluate the impact of $CaCl_2$ treatment in the phenolic profile of tomato fruits. DPPH-online methodology was applied to identify the main phenolic compounds contributing the most to antioxidant activity of the fruits. Glutathione, a tripeptide with a central role in antioxidant system and an indicator of the redox state of the fruits, was also quantified by HPLC-MS. The activity of enzymes involved in the synthesis (γ-glutamyl-cysteinyl synthetase and glutamine synthetase) and recycling of glutathione (glutathione reductase) were evaluated in protein extracts of treated tomato fruits. The obtained results suggest that 1% and 2% $CaCl_2$ (w/v) treatments did not have a significant impact on morphological characteristics of tomato fruits. However, treatment with 6% $CaCl_2$ (w/v) favoured the development of microorganisms. Although 2% $CaCl_2$ (w/v) treatment increased the concentration of some phenolic compounds (particularly tricaffeoylquinic acid), the total phenolic content and antioxidant activity of tomato fruits were not significantly affected by treatments. $CaCl_2$ affected other components of the antioxidant system besides phenolic

compounds. The impact of $CaCl_2$ treatments on enzymes related to the metabolism of glutathione resulted in lower reduced/oxidised glutathione ratio, lowering the redox state of treated tomato fruits. It is further concluded that $CaCl_2$ prevented the degradation of proteins over time, since treated fruits had significantly higher protein content than the controls'.

Key-words

Solanum lycopersicum L., phenolic compounds, antioxidant activity, glutathione, γ-glutamyl-cysteinyl synthetase, glutamine synthetase, glutathione reductase

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Abbreviations and Acronyms

Cys – cysteine

DAD – diode array detector

DPPH – 2,2-diphenyl-1-picrylhydrazyl

d. w. – dry weight

EDTA – ethylenediaminetetraacetic acid

f. w. – fresh weight

GAE – gallic acid equivalents

Glu - glutamate

Gly - glycine

GOGAT – glutamate synthetase

GR – glutathione reductase

GSH – reduced glutathione

GSH-S – glutathione synthetase

GSSG – oxidized glutathione

HPLC – high performance liquid chromatography

MS – mass spectrometry

PAR – photosynthetically active radiation

PCs – phenolic compounds

PMSF – phenylmethylsulfonyl fluoride

PVPP - polyvinylpolypyrrolidone

ROS – reactive oxygen species

SD – standard deviation

TCA – trichloroacetic acid

TE – Trolox equivalents

TEAC – Trolox equivalent antioxidant capacity

TPC – total phenolic compounds

γ-EC – γ-glutamylcisteine

γ-ECS – γ- glutamylcisteine synthetase

Introduction

1. *Solanum lycopersicum* L.

1.1. Classification

Tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family, which includes more than 3000 species, including potato (*Solanum tuberosum*), peppers (*Capsicum spp.*), eggplant (*Solanum melongena*) and tobacco (*Nicotiana tabacum*) [2-4].

Tomato plants are perennial, with 1 to 3 meters in height and a weak stem that often sprawls over the ground and may vine over other plants [5]. Bright yellow flowers (Fig 1), and pinnate or pinnatifid, non-spiny leaves distinguish tomato and its close relatives from other *Solanum* species [6].

Figure 1. *S. lycopersicum* cv Micro-Tom flower

According to botany, tomato is fruit, however, it has much less sugar than other fruits, so for culinary purposes it is considered a vegetable [3,5].

1.2. Production and economic value

Originally from South America, tomato was brought to Europe in 16th century and then distributed worldwide [2,6]. In the 18th and 19th centuries it was intensely domesticated, and since the 20th century plant breeding resulted in several morphological different *S. lycopersicum* cultivars. Currently there are varieties with all shapes, colors, and sizes [2,6].

Nowadays, tomato is mostly produced in temperate zones with long summers and winter precipitation or in (sub)tropical climates. Tomato production, either in open field or in greenhouses, has been greatly increasing in the last fifty years, rising from 27.6 million tons in 1961 to 171 million tons in 2014, with expectations to increase in the next years. Sixty % of this production occurred in Asia, 15% in Americas and 13% in Europe, with China, India, USA, Turkey and Egypt being the top 5 tomato producers. Between the EU countries, the largest 3 producers are Italy, Spain, and Portugal. Top 5 tomato consumers are China, European Union, Mediterranean Africa, and North America Free Trade Agreement (NAFTA) countries (Canada, Mexico, USA). Tomato has

also a great economic importance, since fresh tomato export achieved 7.1 billion euro in 2015 and it is economically the most important in the vegetable category [4,7].

In 2015, Portugal had a cultivated area of 19,300 ha, producing 1.4 million tons of tomato, mainly for export. In the national territory, production is focused in Ribatejo and Alentejo [4].

1.3. Organoleptic and nutritional qualities

Tomato is consumed world-wide, fresh or processed, being an ingredient of many dishes, salads, sauces, ketchup, juices, purees or soups [3,4]. Besides its organoleptic properties, such as its taste, aroma and texture, tomato is appreciated for its low caloric supply, low-fat, relatively high fiber content and a variety of phytochemicals with health benefits [3,5,8].

It is known that diet has an important role in human health, particularly fruits and vegetables. Consumption of tomato and its products, such as sauces, ketchup and pizza have been associated with reduced risk of cardiovascular diseases, cancer, macular degeneration, osteoporosis, cognitive dysfunction, and ultraviolet light-induced skin damage [5,7,9]. These health benefits are attributed to a series of compounds with antioxidant activity present in tomato, in particular carotenoids like lycopene and β-carotene and phenolic compounds. When talking about the chemical composition of tomatoes it is also important to highlight some other compounds: vitamins A, B and C, phytosterols (that help control cholesterol levels), folic acid, and minerals like iron and phosphorous [5,7,9,10].

1.4. Tomato as a model plant: *Solanum lycopersicum* cv Micro-Tom

Tomato has a set of characteristics that make it an excellent model plant. Tomato, a diploid plant with 2n=24 chromosomes, has a relatively small genome, already sequenced, with 950 Mb. Also, it has sympodial shoots and compound leaves, lacks gene duplication, has high self-fertility and homozygosity, easily controllable pollination and hybridization. Additionally, it has the ability

Figure 2. *S. lycopersicum* cv Micro-Tom fruits

for asexual propagation by grafting, and it is possible to regenerate whole plants from different plant parts [2,3,11].

Although it was produced for ornamental purposes, *Solanum lycopersicum* cv Micro-Tom (Fig. 2) is a tomato cultivar widely used as a model plant thanks to its small size (10-20 cm height), short-life cycle (70-90 days) and high efficiency of genetic transformation [3,7,12]. Additionally, *S. lycopersicum* cv Micro-Tom can be grown at high density in normal growth chambers under controlled conditions, which is especially important for evaluating plant responses to environmental factors and mineral nutrition [7].

2. Phenolic compounds

2.1. Definition and function

Phenolic compounds (PCs) are a complex group of more than 8000 compounds that contain one phenol group (an aromatic ring with a hydroxyl functional group) [13,14]. These products, resulting from secondary metabolism, can be found in all plant parts, usually in glycosylated forms [1,14].

Even though PCs are secondary metabolites, they play an important role in plant survival. Besides being involved in mechanical support and attraction of pollinators and fruit dispersers, PCs absorb damaging ultraviolet radiation, are involved in defense against herbivores and pathogens, and in allelopathy processes [13].

2.2. Classification

2.2.1. Phenolic acids

Phenolic acids are divided in hydroxybenzoic acid derivatives and hydroxycinnamic acid derivatives according to the size of the carbon chain attached to the carboxyl group appended. Different derivatives result from different hydroxylation and methylation patterns of the aromatic ring. In plants, hydroxycinnamic acids are the most common [15,16].

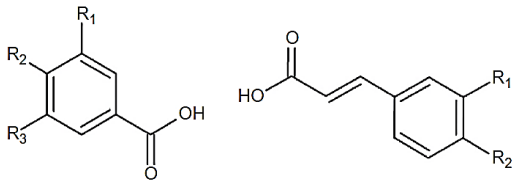


Figure 3. Basic structures of phenolic acids (hydroxyl-benzoic and -cinnamic acids) [15]

Caffeic, *p*-coumaric and ferulic acids are the most common hydroxycinnamic acid derivatives, while *p*-hydroxybenzoic, vanillic and protocatechuic acids are the most common hydroxybenzoic acid derivatives.

Phenolic acids are found throughout all plant kingdom, usually bound to simple or complex carbohydrates, organic acids, flavonoids or terpenoids, and it is estimated that humans consume about 1-2 g of phenolic acids per day, depending on the quantity of plant-based food on their diet [15,16].

These compounds are reported to have antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory, antimicrobial, and anti-obesity effects [15,16].

2.2.2. Flavonoids

Flavonoids are a family of more than 6000 phenolic compound that share a basic structure of two (A, B) phenolic rings linked to a third ring (C), heterocyclic pyran or pyrone, and all three rings are hydroxylated and methylated at different levels [15,16]. Usually they are found in plants bound to sugars (mono-, di- or tri- saccharides), resulting in an enormous variety of structures. Flavonoids can be divided in six groups (flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavonoids) that even though they share the same backbone structure, exhibit unique functional characteristics. [15]. In general, flavonoids antioxidant activity increases with more hydroxyl substituents but decreases with glycosylation.

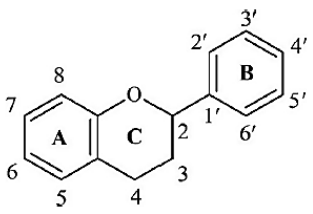


Figure 4. Generic and basic structure of a flavonoid molecule [15].

Both flavonols and flavones can be found in foods in their aglycone forms. While flavones have a double bond between positions 2 and 3 of C ring, flavonols have a hydroxyl group at position 3. Flavanols are also usually found in its aglycone form or esterified with gallic acid [15,16].

Flavanones, found almost exclusively in citric fruits, constitute another flavonoid group without substituents at the position 3, but differ from flavones by the absence of the double bound between C-2 and C-3 [15-17].

Anthocyanidins, flavonoids with an hydroxy group at the position 3, are the aglycone forms of anthocyanins, pigments that give colors from red to purple to plant and can be found mainly in fruits [15,16].

In isoflavonoids, mainly found in the Leguminosae family, the B-ring is bound to C-3 position instead of C-2 like in other flavonoids [15,16].

Besides the antioxidant capacity, flavonoids are reported to have antipyretic, anti-inflammatory, anticancer, antibacterial and antifungal activities, as well as chemopreventive, antiproliferative, antidiabetic and neuroprotective properties. Cardiovascular activity may also benefit from flavonoids antiaggregatory, vasodilator, antihypertensive, antifibrotic and antihypercholesterolemic properties. The digestive system can be protected by flavonoids with antisecretory and antidiarrheal properties.

2.2.3. Stilbenes

Stilbenes are a group of phenolic compounds with a 1,2-diphenylethylene backbone, synthesized by plants in response to infection or injury [18,19]. *Trans*-resveratrol is the basic unit of most stilbenes in plants.

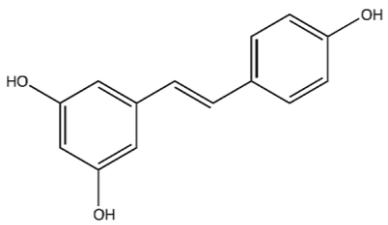


Figure 5. Structure of *trans*-resveratrol [15].

Stilbenes are found manly in grapes, red wine, and peanuts, although present in extremely low levels in human diet [16,19].

Resveratrol can be used to maintain nutritional quality and increase pharmaceuticals shelf life because it delays the formation of toxic oxidation products [16].

Stilbenes are antioxidative, anticarcinogenic, antitumoral, which makes them capable of delaying or inhibit several animal diseases as cardiovascular diseases and cancer [16]. Resveratrol is also used in obesity treatments [20].

2.2.4. Coumarins

Coumarins are a group pf phenolic compounds consisting of a benzene ring condensed with a heterocyclic α-pyron ring and can be divided in four categories: simple coumarins, furanocoumarins, pyranocoumarins and substituted coumarins [15].

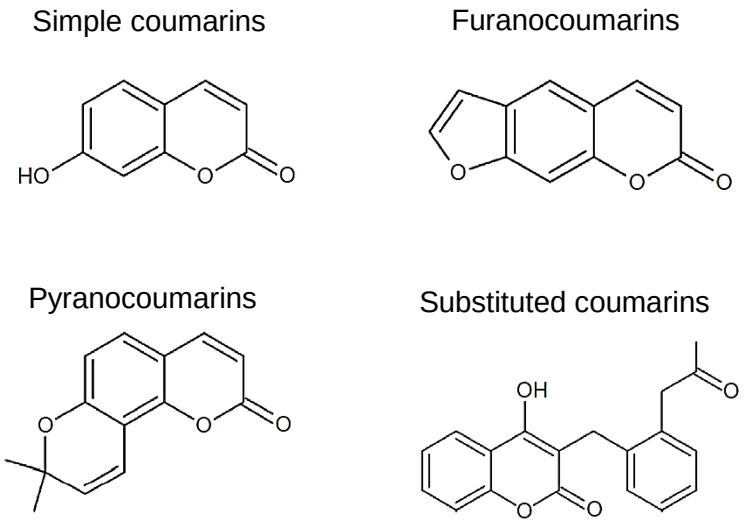


Figure 6. Structure of different categories of coumarins [15].

In simple coumarins the benzene ring is bound to a hydroxyl, alkoxy or alkyl group. Furanocoumarins may be linear, with a furan ring bound to carbon C6 or C7, or angular, with another furan ring attached to carbon C7 or C8 of a benzo-α-pyrone [15]. Pyranocoumarins are similar to furanocoumarins but contain a six-membered ring [21]. Substituted coumarins have substituents on carbon C3 and C4 [15].

Coumarins are found commonly in Angiosperms, in cinnamon, cassia leaf and lavender oils, as well as in fruits, green tea, and chicory [15,22]. They are reported to have anticoagulant, anti-inflammatory, antimicrobial, anticancer, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant,

vasodilating, anthelmintic, analgesic, estrogenic, sedative, neuroprotective and hypothermic properties [15,23].

2.2.5. Lignans

Lignans are formed by two phenylpropane units bonded by a C-C link between the central atoms of the corresponding side chains (at position 8 or β), bound known as β- β'. When 3-3', 8-*O*-4', or 8-3' bounds occur, compounds are classified as neolignans [15].

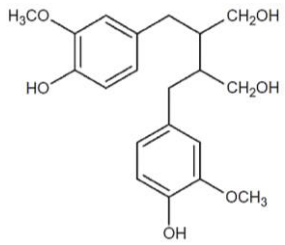


Figure 7. Basic structure of lignans [15].

According to the way in which oxygen is incorporated into the compound's skeleton and the cyclization pattern, lignans can be divided in 8 groups: furofuran, furan, dibenzylbutane, dibenzylbutyrolactone, aryltetralin, arylnaphthalene, dibenzocyclooctadiene, and dibenzylbutyrolactol. The variety of lignans is increased by variations in oxidation levels of the aromatic rings and the propyl side chains, as well as by the existence of different enantiomers [15,24].

The major source of lignans is flaxseed, with much higher concentration of lignans than pumpkin seeds, sesame rye, cranberry, and black and green tea [15].

Lignans have anticarcinogenic, antitumoral, antimitotic, antioxidant, antimicrobial, anti-inflammatory and immunosuppressive activity, and can prevent cardiovascular diseases and type II diabetes [15,25].

2.2.6. Tannins

Depending on their structures, tannins can be divided in two groups: hydrolysable tannins and condensed tannins, also known as proanthocyanidins. There are also some

compounds that have been recognized as tannins and that do not belong to those two groups, such as phlorotannins (obtained from brown algae) and caffeates [15,16].

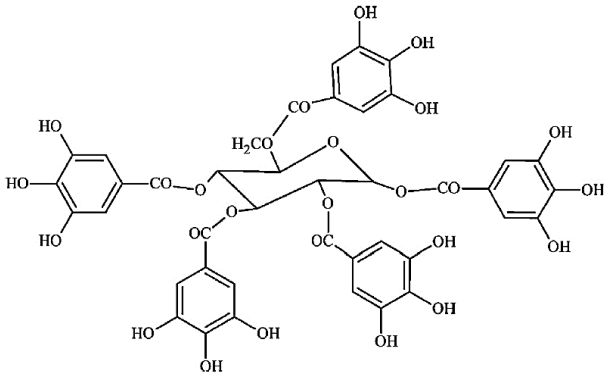


Figure 8. Structure of tannic acid [15].

Condensed tannins are oligomers and polymers of flavonoids, specifically flavan-3-ols, while hydrolysable tannins result from esterification reactions of nonphenolic compounds (like gallic acid and ellagic acids) bonded to a central polyol core, usually glucose or other carbohydrate [15,16]. Hydrolysable tannins can be hydrolyzed by weak acids, bases, or appropriated enzymes, producing carbohydrate and phenolic acids [15,26]

Tannins are known to be anti-nutritional because their phenolic groups bind very tightly to -NH groups of peptides and proteins, avoiding their hydrolysis and digestion in the stomach [16]. Tannins are known to give astringency and bitterness to a variety of foods [15].

Tannins can be found in fruits, nuts, and beverages, highlighting red fruits, beer, wine, and tea [15,16] .

Tannins can be used to treat skin inflammation and injuries and in prevention or delaying of chronic degenerative diseases [15]. These compounds are also reported to have antiproliferative, antimicrobial, antioxidant, and antimutagen properties [15,16].

2.3. Biosynthesis of phenolic compounds

Phenolic compounds may be synthesized by the malonic acid pathway or by the shikimic acid pathway. While malonic acid pathway is very important in fungi and bacteria, in plants the majority of PCs come from the shikimic acid pathway. This pathway is responsible for synthesizing aromatic amino acids (phenylalanine, tyrosine,

and tryptophan) from simple carbohydrates precursors derived from glycolysis and the pentose phosphate pathway. Phenylalanine ammonia lyase (PAL) catalyzes the elimination of an ammonia molecule from phenylalanine originating cinnamic acid and C6-C3 structures (Fig 9) and then occurs the addition of substituents, such as hydroxyl groups. These C6-C3 structures (a benzene ring with a side chain of 3 carbons) are phenylpropanoids and are used like building blocks for the synthesis of others phenolic compounds [13,14].

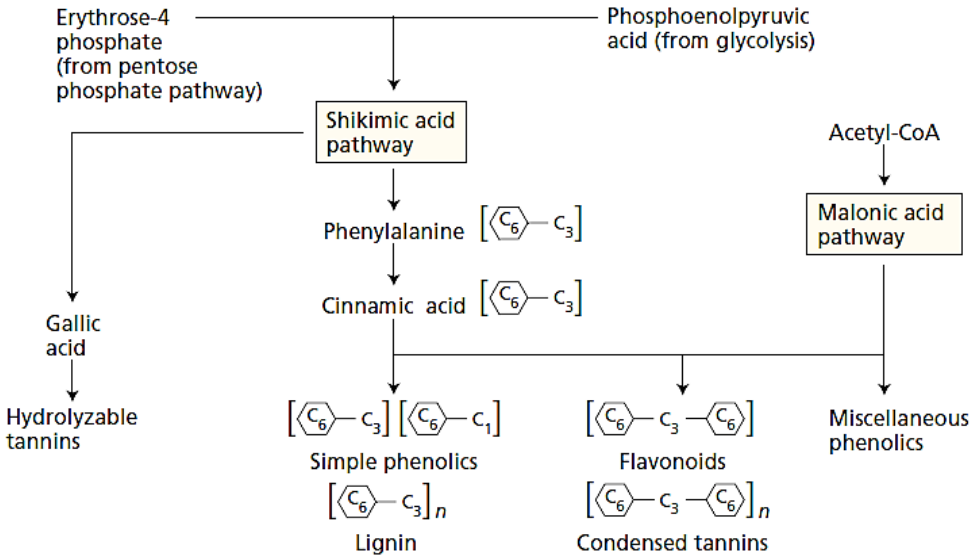


Figure 9. Schematic representation of phenolic compounds biosynthesis in plants [13].

2.4. Phenolic compounds in food

Phenolic compounds are determinants for odor, taste, astringency, and color, having a major role in organoleptic characteristics of a wide variety of foods [15]. Fruits, vegetables, cereals, oilseeds and plant oils, tree nuts, herbs and spices and even drinks like tea and wine all have PCs in their composition [14,16]. Table 1 resumes dietary sources of different phenolic compounds classes [27] .

Table 1. Dietary sources of plant phenolics [16].

Phenolic compounds	Dietary source
Phenolic acids	
Hydroxycinnamic acids	Apricots, blueberries, carrots, cereals, pears, cherries, citrus fruits, oilseeds, peaches, plums, spinach, tomatoes, eggplants
Hydroxybenzoic acids	Blueberries, cereals, cranberries, oilseeds
Flavonoids	
Anthocyanins	Bilberries, black and red currants, blueberries, cherries, chokecherries, grapes, strawberries
Chalcones	Apples
Flavanols	Apples, blueberries, grapes, onions, lettuce
Flavanonols	Grapes
Flavanones	Citrus fruits
Flavonols	Apples, beans, blueberries, buckwheat, cranberries, endive, leeks, lettuce, onions, olive, pepper, tomatoes
Flavones	Citrus fruits, celery, parsley, spinach, rutin
Isoflavones	Soybeans
Xanthones	Mango, mangosteen
Tannins	
Condensed	Apples, grapes, peaches, plums, mangosteens, pears
Hydrolyzable	Pomegranate, raspberries
Other phenolics	
Alk(en)ylresorcinols	Cereals
Arbutin	Pears
Avenanthramides	Oats
Capsaisinoids	Pepper
Coumarins	Carrots, celery, citrus fruits, parsley, parsnips
Lignans	Buckwheat, flaxseed, sesame seed, rye, wheat
Secoiridoids	Olives
Stilbenes	Grapes

2.5. Phenolic compounds in *S. lycopersicum*

As stated above, phenolic compounds are among the main responsible for the health benefits associated with tomato consumption [5,7,9,15,16]. Thus, it is important to know which of these compounds are specifically found in tomatoes.

The cultivar, cultivation conditions (light, temperature, soil, fertilization), ripeness at harvest, as well as handling and storage methods all influence metabolic composition of tomato fruits [28-30]. Metabolic profile can also differ between individual fruits and even between tissues of the same tomato [1,8,31-34].

Phenolic acids are the most found class of phenols in tomato, in particular hydroxycinnamic acids and derivatives, such as ferulic, caffeic, coumaric and chlorogenic acids [1,7,35,36].

The flavonoids are the other main class of phenolic compounds present in tomato fruits, represented mainly by chalconaringenin, naringenin, quercetin, rutin, kaempferol and their derivatives [1,16,35,36]. However, in some varieties, naringenin or its glycosylated derivatives were not found, which was interpreted as a characteristic of those varieties [35].

Resveratrol and piceid, two stilbenoids, were also found in tomato fruits, along with their isomers [1].

2.5.1. Location of phenolic compounds in *S. lycopersicum*

Phenolic compounds concentration varies greatly in the different parts of the tomato, with higher concentrations in the epidermal and placental tissues [1].

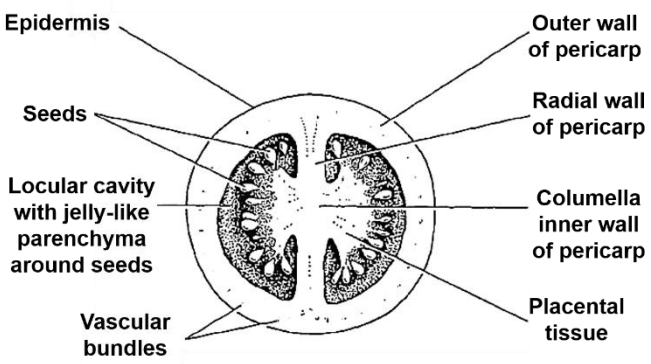


Figure 10. Transverse section of mature tomato fruit showing the main anatomical features [1].

Regarding flavonoids, 95-98% of chalconaringenin and 98% of total flavonols are found in the skin or epidermal tissues, while chalconaringenin, naringenin and the trisaccharides of kaempferol and quercetin exist not only in the epidermis but also in the vascular regions [1].

Phenolic acids are distributed more evenly in tomato fruits, with some hydroxycinnamic acids (caffeic, coumaric, ferulic, chlorogenic, di-caffeoylquinic, and tri-caffeoylquinic acids) derivatives being found in all tomatoes' tissues. However, different isomers of the same phenolic acid can have differential distribution within the fruit, with some being more abundant in the vasculature region and others in the jelly parenchyma or, like flavonoids, in the epidermis [1].

3. Glutathione

3.1 Structure

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is a thiol tripeptide found in the majority of organisms, including plants [37]. The sulphydryl group (-SH) makes glutathione more reactive and a powerful reducing agent. Additionally, the rare bond between the amino acid group of cysteine and the γ-carboxyl group of glutamic acid can protect glutathione from hydrolysis by peptidases [38,39].

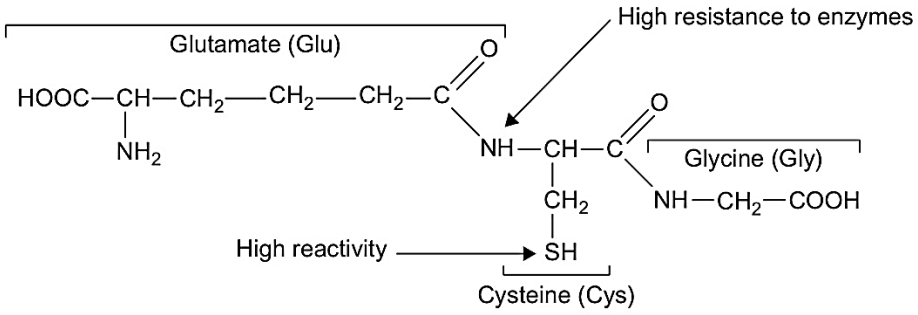


Figure 11. Chemical structure of glutathione [39]

Glutathione is the principal thiol in plant cells and can be found at millimolar concentrations, with an average ratio reduced:oxidized (GSH:GSSH) of at least 20:1, at normal conditions [40]. High alterations in this ratio are an indicator of stress [37].

3.2. Function

Glutathione is involved in several plant processes, such as detoxification of xenobiotics, herbicides, air pollutants and heavy-metals, tolerance to abiotic stresses (salt and drought stress or chilling damage), nonetheless, its principal function is in redox homeostasis and signaling, since it is involved in direct and indirect control of reactive oxygen species (ROS) concentrations [38-40]. As an antioxidant, glutathione is oxidized to glutathione disulphide (GSSH) that is after recycled to GSH by glutathione reductase (GR; EC 1.8.1.7) [38,39]. Glutathione has an important role as electron donor in the ascorbate-glutathione cycle, regenerating ascorbate from dehydroascorbate and, at the same time, removing H_2O_2, harmful to cells [41].

3.3. Glutathione biosynthesis

In plants, GSH synthesis occurs in two ATP-dependent steps. First, γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) catalyzes the reaction between cysteine and glutamate, originating γ-glutamylcysteine (γ-EC). Then, by the action of glutathione synthetase (GSH-S; EC 6.3.2.3), glycine is added to γ-EC.

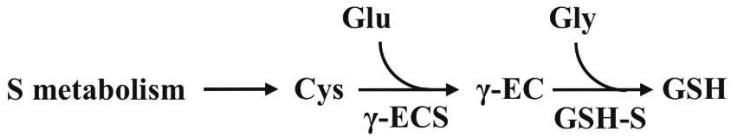


Figure 12. Simple model of glutathione synthesis. γ-ECS: γ- glutamylcysteine synthetase; γ-EC: γ- glutamylcysteine; GSH-S: glutathione synthetase [38].

γ-ECS activity and cysteine concentration are the major limiting factors for GSH synthesis, since among the three amino acids used in GSH synthesis is usually the one found in lower concentrations. Besides, cysteine is the first organic product of sulfur assimilation, so GSH synthesis is also dependent on sulfur availability and its metabolism [37,40,42].

Additionally to S availability, N availability can also limit the biosynthesis of GSH and therefore, another important enzyme for glutathione synthesis is glutamine synthetase (GS; EC 6.3.1.2). By GS action, inorganic nitrogen is assimilated in the form of ammonium (NH_4^+) and, together with glutamate, generates glutamine at the expense of ATP. Then, glutamine and 2-oxoglutarate generate 2 glutamate molecules in a reaction catalyzed by glutamate synthase (GOGAT; EC 1.4.1.13). The glutamate produced by the GS-GOGAT cycle can be incorporated into other amino acids or used as substrate for γ-ECS in GSH synthesis [43].

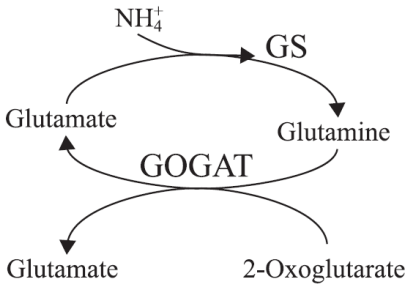


Figure 13. GS-GOGAT cycle [43].

4. Fruits and vegetables quality

4.1. Quality

Fruits and vegetables quality results from a dynamic between physicochemical properties and the perception of the consumer [44]. Quality depends on intrinsic attributes such as genotypic, agroenvironmental or post-harvest factors as well as on socioeconomic and marketing factors (extrinsic characteristics) [45]. While product-oriented definition of quality is preferentially based on appearance and shelf life quantifiable traits, consumer-oriented definition of quality is based on the consumer's needs [46]. However, neither of these approaches alone give a correct definition of quality [44].

4.2. Post-harvest technology and quality maintenance

The advances in post-harvest physiology and technology allowed the improvement of chemical, physiological and physical quantification methods and, consequently, the knowledge about fruits and vegetables quality [47], with special attention to how ethylene controls fruit ripening processes and how it influences fruit and vegetable physiology, quality and post-harvest life [48]. Post-harvest practices and technologies are applied for the maintenance of quality of the products along with the supply chain and cannot modify their potential quality resulting from pre-harvest factors. This means that improvements on products quality results from manipulation of the ripening process and not from alterations on inherent fruit and vegetables characteristics [44,49]. Application of post-harvest technologies on climacteric commodities intent to decrease, or even inhibit, ethylene production and the climacteric peak in respiratory activity, delaying ripening and consequent senescence [44]. Post-harvest treatments may affect physiological processes linked to ripening, making the determination of ripening stage and product quality based on solitary indices deceitful, which may result in a discrepancy between appearance-based and flavor-based shelf life [50].

4.2.1. Post-harvest treatments

Great percentage of fruits and vegetables production is lost due to unsatisfactory preservation and handling processes. Other major causes of deterioration are desiccation, pathogen attacks, physiological disorders, respiration rate, ethylene effect,

rate of compositional changes, water stress and mechanical injuries. Temperature, relative humidity, atmospheric composition, and sanitation are the major external factor that affect biological decay progress. [47,51,52]. Moreover, physical damage, extended storage duration, high temperatures, low relative humidity, and chilling injury are responsible for post-harvest nutritional losses in fruits and vegetables [47].

In order to avoid post-harvest damages and loss of nutritional quality, several treatments may be applied to fresh horticultural crops: cleaning and removal of excess surface moisture, waxing and application of other surface coatings, hot treatments, fumigation, application of fungicides or special chemical treatments. The manipulation of the environment during storage of fruits and vegetables can also decrease their spoilage. Decay rates can be reduced by controlling air movement, air exchange or ventilation, exclusion or removal of ethylene, use of controlled or modified atmospheres, use of package inserts and effective water disinfection along with further sanitary procedures [50].

4.2.1.1. Calcium post-harvest treatments

Calcium chloride ($CaCl_2$) is among the several substances used in post-harvest treatments, with the aim to maintaining fruits and vegetables quality and increase their shelf life by slowing or inhibiting physiological processes leading to ripening and fruit decay [10,53-60].

$CaCl_2$ is reported to decrease ethylene synthesis, one of the major responsible for the beginning of the ripening processes and to delay color development [58,61,62].

In addition, other described effects of $CaCl_2$ post-harvest treatments application is a decrease in weight loss and higher firmness in treated fruits. This is explained by a maintenance of cellular wall structure when calcium binds to its components, namely pectin. Calcium bonds with cellular wall components, maintaining its structure, and reducing water loss and transpiration rates [60,62,63]. A decrease in activity of enzymes related with ripening and fruit softening (polygalacturonase and pectin methyl esterase) is also described as a consequence of $CaCl_2$ treatments [64-67].

Additionally, $CaCl_2$ reduces the loss of phenolic compounds and ascorbic acid (vitamin C) and does not affect beta-carotene concentration, so it may be used for the

maintenance of the nutrient profile during storage and defense against microorganisms, avoiding quality loss during storage [53,68].

$CaCl_2$ post-harvest treatments have been studied in tomato fruits and the above effects have been observed, however, several CaCl2 concentrations (in the range 1-6% (w/v)) are recommended by different studies as the ideal ones for maintaining tomato quality during storage [10,54-56].

Aims

This work intends to clarify and extent the existing knowledge about the effects of calcium chloride post-harvest treatment in nutritional quality of tomato fruits.

Previous studies of $CaCl_2$ post-harvest treatments recommend different concentrations as the ideal ones for application on tomato fruits, therefore in this work a wide range of $CaCl_2$ concentrations is used, with the aim of clarifying this question.

One of the other main goals of this work is to find out the effects $CaCl_2$ post-harvest treatment may have in tomato phenolic compounds as well as in glutathione concentration and in the activity of enzymes related with its metabolism. These analyzes will give new information about the antioxidant defense system during storage time in treated tomatoes treated with $CaCl_2$.

Methods and materials

Plant material and growth conditions

Certified *Solanum lycopersicum* L. cv. Micro-Tom (Tomato Genetics Resource Center (TGCR); germplasm LA3911) seeds were used in all assays in this study. The seeds were surface-sterilized with 70% ethanol for 10 minutes, followed by 20% commercial bleach containing 0.02% tween-20 for 5 min, in constant agitation. Then, the seeds were washed several times with sterilized double-distilled water under constant agitation and left to dry on filter paper. Afterwards, seeds were sown in Petri dishes (10 cm diameter) with 1x Hoagland solution (HS) solidified with 0.625% (w/v) agar [13]. The Petri dishes were placed for two days at 4ºC for seed stratification (to synchronize the seed germination), and then transferred to a growth chamber (16 h light/ 8 h dark) at 25ºC, with a photosynthetically active radiation (PAR) of 60 µmol m^{-2} s^{-1} for 12 days. After this period, seedlings were cultivated in individual pots with a typical greenhouse soil and maintained in a growth chamber (16 h light/ 8 h dark) at 21ºC with PAR of 120 µmol m^{-2} s^{-1}, watered with tap water.

For the optimization of extraction conditions cherry tomatoes bough at a local grocery store were used.

Sampling

S. lycopersicum L. cv. Micro-Tom fruits were harvested at their red stage (more than 90% of surface showing red color). The fruits were divided into 7 groups, according to the table below. Distribution of tomatoes was made assuring the groups were uniform in terms of size and mass of the fruits.

Table 2. Group distribution of *S. lycopersicum* L. cv. Micro-Tom fruits

	t0	1 week	2 weeks
Control			
1% $CaCl_2$			
2% $CaCl_2$			

$CaCl_2$ treatments

Each group of tomatoes was dipped for 30 minutes in 200 mL of treatment solution (1%, 2%, and 6% (w/v) $CaCl_2$ anhydrous [Panreac, Barcelona]), with constant agitation, at room temperature. Control groups were dipped in ultrapure water. The fruits were left at room temperature until completely dried, weighted, and then moved to a growth chamber, stored in boxes, with ventilation holes and no direct light. After the time of each treatment (1 or 2 weeks), fruits were collected, weighted again and either frozen at -20 ºC and freeze-dryed or ground in liquid nitrogen (N_2) and frozen at -80 ºC for subsequent analyses.

Weight loss

Each group of fruits was weighted before storage and then again at the end of the storage period (1 or 2 weeks). Percentage of weight loss was calculated using the equation below:

$$\%\ weight\ loss = \left(\frac{Initial\ sample\ weight - Final\ sample\ weight}{Initial\ sample\ weight}\right) \times 100$$

Extract preparation

To extract phenolic compounds from treated fruits, 20 mL of methanol 70% (v/v) were added to 1 g of freeze-dried tomato ground to powder with a mortar and pestle. This mixture was exposed to ultrasounds for 60 minutes and centrifuged (6,000 g for 15 min at 10ºC). Supernatants were vacuum-filtered and stored at 4ºC.

These extraction conditions were selected after an optimization process in which different volumes (10, 20 and 40 mL) of different solvents (methanol, ethanol, acetone and water) were tested. Different proportions solvent:water (50:50, 70:30, 100:0 (v/v)) were evaluated, as well as different times of exposure to ultrasounds (15, 30 and 60 minutes).

Total phenolic compounds (TPC) quantification

The content of total phenolic compounds in tomato extracts was determined by the Folin-Ciocalteau method adapted to microplates, according to Horszwald and Andlauer (2011) . Fifteen μL of each sample (extracts or standards) was mixed with 240 μL of Folin-Ciocalteau reagent (diluted in water, 1:15). The plate was protected from the light for 10 min and then 15 μL of 20% (w/v) sodium carbonate was added to stop the reaction. The microplate was automatically shaken for 3 seconds before measuring the absorbance at 750 nm. Gallic acid in the range of 25 to 700 mg L^{-1} was used as a calibration standard. The results were expressed in Galic Acid Equivalents (GAE) mg g^{-1} dry weight.

Antioxidant capacity determination by DPPH method

The antiradicalar capacity of each extract was evaluated by a microplate-adapted colorimetric method according to [69]. Twenty μL of the samples (extracts diluted 1/5 in methanol, or standards) were mixed with 300 μL of DPPH• solution (10 mg dissolved in 250 mL methanol). The reaction occurred for 30 minutes, protected from light. The plate was automatically shaken for 3 seconds and the absorbance was measured at 515 nm. Trolox in the range of 17 to 209 mg L^{-1} was used as a calibration standard. Results were expressed in Trolox Equivalents (TE) mg g^{-1} dry weight.

HPLC-DAD-MS^n

The qualitative study of the phenolic composition of tomato fruits extracts was performed by HPLC (high-performance liquid chromatography) coupled with an ion-trap mass spectrometer and diode array detector (DAD), according to Valente (2018), with some modifications. The HPLC system (Thermo Electron Corporation, Waltham, MA)

consisted of a low-pressure quaternary pump with autosampler and a diode array detector (Finnigan Surveyor Plus, Thermo Fisher Scientific). Separations were carried out on a Phenomenex (Torrance, CA) Gemini-NX C18 column (150 mm x 4.6 mm; 3 µm) and a guard column (4 mm x 3.0 mm). The chromatographic conditions were the following: flow rate 0.4 mL min^{-1}, sample injection volume 20 µL, and a binary mobile phase (A, methanol and B, 0.1% aqueous formic acid). A gradient program was used as follows: 0 to 40 min, linear increase from 10% to 30% of A, 40 to 60 min, increase to 45% of A, 60 to 90 min, linear increase to 100% A and conditions maintained for 5 min; return to initial conditions in 15 min and conditions maintained for 10 min before the next injection. A quadrupole ion-trap mass spectrometer (Finnigan LCQ Deca XP Plus) coupled with an electrospray ionization (ESI) source was used. The interface conditions were applied as follows: capillary temperature, 325 ºC; source voltage, 5.0 kV; capillary voltage, -15.0 V; tube lens voltage, -30 V; sheath gas (N_2) flow at 60 arbitrary units and auxiliary gas (N_2) flow at 23 arbitrary units. Data acquisition was performed in the range 160-1000 *m/z*. The negative ion polarity mode was selected due to a better selectivity in comparison with positive ion mode. Tandem mass spectrometric studies were performed (MS^2 and MS^3). For the MS^n analyses activation energy of 45% was applied. The pseudomolecular ions were fragmented by collision-induced dissociation (CID) with the nitrogen collision gas in the ion trap. Diode array detection was conducted by scanning between 190 and 600 nm.

Phenolic compounds were characterized and identified according to their UV and mass spectra. Quantification of phenolic compounds was performed in gallic acid equivalents (mg GAE g^{-1}) at 280 nm. Data acquisition and processing was achieved by using Xcalibur software version 2.1.0 (Finnigan, San Jose, CA).

Online HPLC-DPPH analysis

Screening of the radical scavenging activity of tomato fruits extracts was performed using a HPLC-DPPH online methodology, specially assembled and optimized in our laboratory. This methodology is based on a post-column reaction of the antioxidant with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, which is monitored in real time (Fig 1) [71].

Separation was carried out using a HPLC-DAD system (Jasco Corporation, Tokyo, Japan) consisted of a low-pressure quaternary gradient unit (model LG-1580-04)

and an autosampler (AS-950). Separations were achieved on a Gemini C18 column (150 mm x 4.6 mm; 3 µm particle size) and a guard column (4 mm x 3.0 mm) from Phenomenex (Torrance, CA), using a binary solvent gradient (A, methanol and B, 0.1% formic acid in water) at 0.4 mL min^{-1} set as follows: 0 to 40 min, linear increase from 10% to 30% of A, 40 to 60 min, increase to 45% of A, 60 to 90 min, linear increase to 100% A and conditions maintained for 5 min; return to initial conditions in 15 min and conditions maintained for 10 min before the next injection. Sample injection volume was 20 µL. Online radical scavenging capacity of individual compounds was evaluated by post-column reaction using a "T" form connection with DPPH reagent (10 µM DPPH solution methanol:0,1% formic acid in water, 1:1, v/v, prepared from a stocking solution of 1.3 mM DPPH in methanol) supplied by a second HPLC isocratic pump (Hewlett Packard Series 110, Waldbronn, Germany) at a flow rate of 0.4 mL min^{-1}. The sample DPPH-mixture was passed through a 20 m PTFE reaction coil (inner diameter 0.5 mm, outer diameter 1.6 mm, Kinesis, UK) before measurement at 280 and 518 nm using a photodiode array detector (model MD-1510 UV/Vis multiwavelength detector) scanning between 200 and 600 nm. The mixture was protected from light during the reaction. Data processing was made using ChromNAV software version 2.02.01 (Jasco Corporation, Tokyo, Japan).

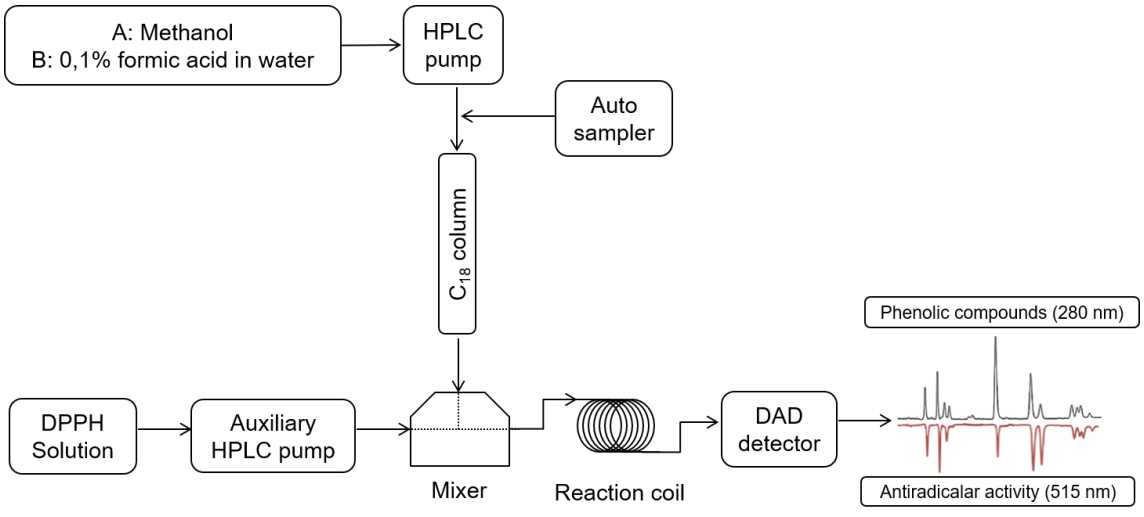


Figure 14. Schematic representation of HPLC-DPPH online methodology [72].

Glutathione quantification

Both reduced (GSH) and oxidized (GSSH) glutathione forms were quantified according to Pinto (2016), with some modifications. 0.5 g of tomato fruits were homogenized with 1.5 mL of 3% (w/v) meta-phosphoric acid and quartz sand at 4ºC. Extracts were centrifuged at 19,000 g for 15 minutes, at 6ºC and supernatants were collected and filtered with a 0.45 µm nylon filter.

Quantification of GSH and GSSG was performed by HPLC coupled with an ion-trap mass spectrometer and diode array detector (DAD). The HPLC system (Thermo Electron Corporation, Waltham, MA) consisted of a low-pressure quaternary pump with autosampler and a diode array detector (Finnigan Surveyor Plus, Thermo Fisher Scientific). Separations were carried out on a Phenomenex (Torrance, CA) Gemini-NX C18 column (150 mm x 4.6 mm; 3 µm) and a guard column (4 mm x 3.0 mm). The chromatographic conditions were the following: flow rate 0.4 mL min^{-1}, sample injection volume 25 µL, and a binary mobile phase (A, methanol and B, 0.1% aqueous formic acid). A gradient program was used as follows: 0 to 10 min, 10% of A, 10 to 15 min, increase to 50% of A and conditions maintained for 5 min; return to initial conditions in 3 min and conditions maintained for 5 min before the next injection. A quadrupole ion-trap mass spectrometer (Finnigan LCQ Deca XP Plus) coupled with an electrospray ionization (ESI) source was used. The interface conditions were applied as follows: capillary temperature, 275 ºC; source voltage, 4.0 kV; capillary voltage, 31 V; tube lens voltage, 10 V; sheat gas (N_2) flow at 60 arbitrary units and auxiliary gas (N_2) flow at 23 arbitrary units. Data acquisition was performed in selected ion monitoring (SIM) mode, selecting m/z 613 for GSSG and m/z 308 for GSH. Analysis were performed in positive ion mode. The diode array detection was conducted by scanning between 200 and 750 nm. Quantification of reduced and oxidized forms of glutathione was performed using calibration curves established from standard solutions based on the peak area obtained for GSH (5-12,5 mg L^{-1}) and GSSG (1-4 mg L^{-1}) present in the samples. The concentrations of GSH and GSSG were expressed in mg g^{-1} f.w.. Data acquisition and processing was achieved by using Xcalibur software version 2.1.0 (Finnigan, San Jose, CA).

Glutathione reductase activity determination

For the protein extraction, tomato fruits frozen samples (-80ºC) of 500 mg were homogenized with 1.5 mL of extraction buffer containing 100 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, at pH 7.8, quartz sand and 5-10% (w/v) polyvinylpolypyrrolidone (PVPP), under ice-cold conditions. Homogenates were centrifuged at 20,000 g for 20 minutes at 4ºC and supernatants collected and maintained on ice [74].

For the spectrophotometric determination, the following solutions were added to a quartz cuvette: 500 µL of 200 mM potassium phosphate buffer (pH 7.8), 100 µL of 20 mM EDTA, 100 µL of 2 mM NADPH, 100 µM of 5 mM GSSG, 50 µL of H_2O and 150 µL of protein extract. A blank was prepared with extraction buffer instead of protein extract. Absorbance at 340 nm was monitored after the addition of protein extract for 1 minute. GR activity was calculated using the extinction coefficient 6.22 mM^{-1} cm^{-1} and expressed as nkat mg^{-1} protein.

Protein content was determined by the Bradford Method (Bradford, 1976). In triplicate, 75 µL of extract were added to 750 µL of Bradford reagent, and after 15 minutes absorbance was measured at 595 nm. The calibration curve was determined using solutions of bovine serum albumin (BSA) at the range of 20 to 120 µg mL^{-1}.

γ-ECS activity determination

Protein extracts prepared as for the glutathione reductase activity determination were also used for γ-ECS activity determination. The reaction began with the addition of 140 µL of protein extract to 350 µL of reaction mixture (143 mM Hepes, pH 8, 71.43 mM $MgCl_2$, 28.57 mM glutamate. 1.43 mM cysteine, 7.14 mM ATP, 7.14 mM phosphoenolpyruvate, 7.14 mM DTT) and 10 µL of pyruvate kinase (5 U mL^{-1}). A blank was prepared using extraction buffer instead of protein extract, and triplicates were prepared for all reactions. These mixtures were incubated at 37ºC for 45 minutes and the reaction was stopped by addition of 100 µL 50% (w/v) trichloroacetic acid (TCA). After centrifugation for 15 min at 10,000 (6ºC), supernatants were collected, and phosphate content determined by phosphomolybdate method. Protein content was determined by the Bradford Method (Bradford, 1976).

For the phosphomolybdate method [75], 150 µL of color solution (equal parts of 12% (w/v) ascorbic acid in 1M HCl and 2% (w/v) ammonium molybdate tetrahydrate) were added to 50 µL of supernatant. After 20 minutes of incubation at room temperature, 1,000 µL of stop solution (2% acetic acid, 2% (w/v) sodium citrate tribasic dihydrate) were added to stop the reaction, and absorbance was read at 660 nm. A standard curve was constructed using solutions of KH_2PO_4 with concentrations ranging from 0,5 mM to 2,0 mM instead of supernatants (for each know concentration, triplicates were prepared). Results were expressed as nmol PO_4^{3-} min^{-1} mg^{-1} protein.

Glutamine synthetase activity determination

For protein extraction, tomato fruits frozen samples (-80ºC) of 500 mg were homogenized with 0.65 mL of extraction buffer (25 mM Tris-HCl, pH 6.4, 10 mM $MgCl_2$, 1mM DTT, 0.05% Triton X-100, 10% glycerol), quartz sand and 5-10% (w/v) PVPP at 4°C. Extracts were centrifuged (15,000 g, 20 min, 4°C) and supernatants collected.

For GS activity determination [76], 400 µL of reaction mixture (100 mM Trizma base, 125 mM L-glutamine, 157 mM Hydroxylamine, 1.26 mM Manganese (II) chloride ($MnCl_2$), 25 µM Adenosine 5'-diphosphate sodium salt (ADP), pH 6.4) and 50 µL of sodium arsenate were added to 50 µL of extract. The reaction occurred at 30°C and was stopped after 30 minutes with the addition of 500 µL of stop solution (0.16 M Iron (III) chloride ($FeCl_3$), 0.25 M TCA in 37% HCl). The absorbance was measured at 500 nm. Protein content was determined by the Bradford Method (Bradford, 1976), using diluted extracts: 1:10 for controls and 1:1 for treatments. Glutamine synthetase activity was expressed in nkat mg^{-1} of protein.

Statistical analysis

Three technical replicates were produced for each assay, and results were expressed as mean ± standard deviation (SD). To find if there were significant differences between each treatment and respective control, a two-way ANOVA followed by Tukey test for comparison among different means was applied. The statistical analysis of the data was carried out using the software GraphPad Prism 7.00 (GraphPad Software Inc., USA). Differences at $p < 0.05$ were considered significant.

Results

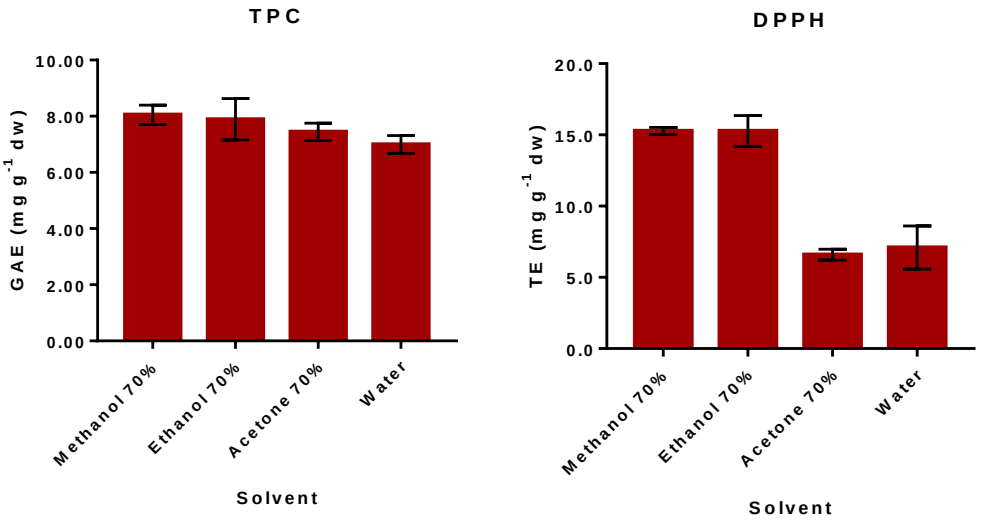
1. Optimization of extraction conditions

1.1. Total phenolic compounds (TPC) quantification and antioxidant capacity determination by DPPH

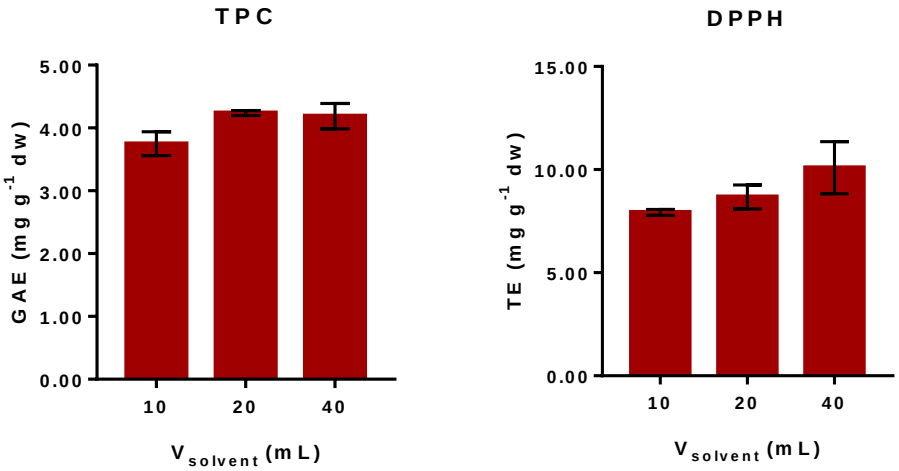
The goal of this optimization process was to select the best conditions to extract the maximum level of phenolic compounds with antioxidant capacity possible. The Folin-Ciocalteu method provides limited information about the phenolic compounds present in the extracts tested, since it only quantifies the total phenolic compounds and does not give information about individual components [77]. The DPPH method is a rapid, simple and inexpensive assay that it is commonly used to determine the overall antioxidant capacity of a variety of samples [78]. Although total phenolic content and antioxidant activity are directly associated, higher amounts of phenolic compounds do not necessarily have higher antioxidant activity, so these results must be evaluated together [16,77].

Methanol and ethanol had similar results in both TPC and DPPH assays (Fig 15 A). Methanol was chosen because it was the solvent of most solutions used in the analytical methods following the extraction, in particular chromatographic separations.

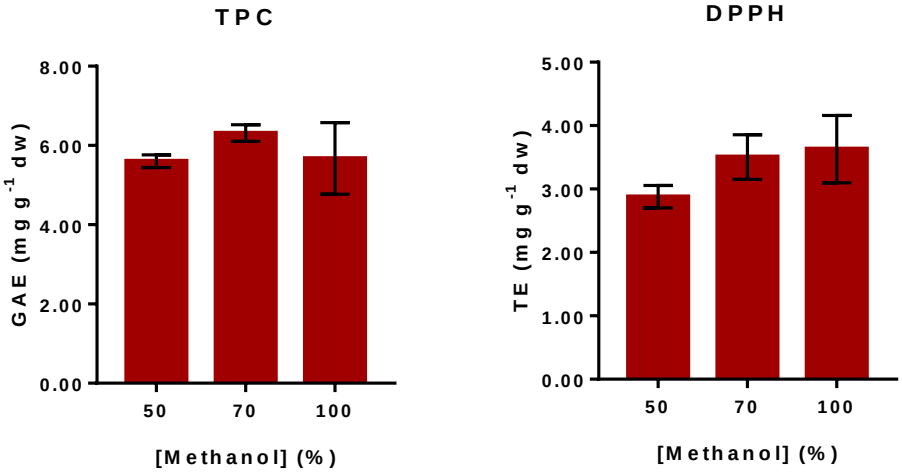
A. 20 mL solvent, 30 minutes



B. Methanol 70% (v/v), 30 minutes



C. 20 mL methanol, 30 minutes



D. 20 mL methanol 70% (v/v)

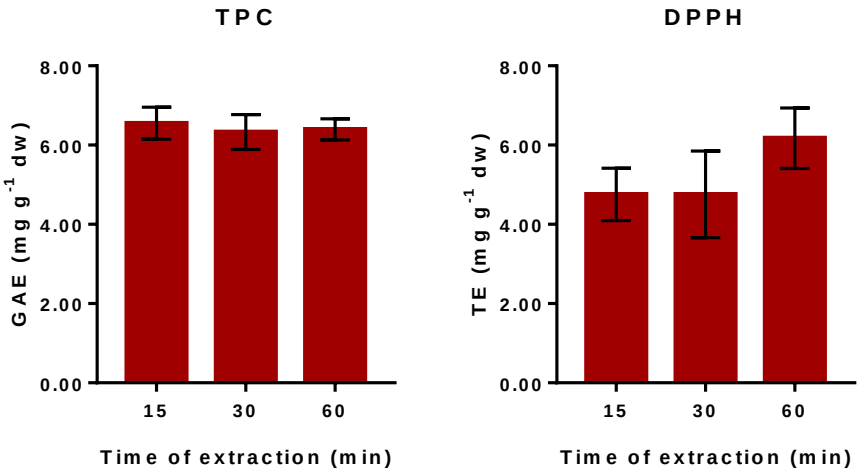


Figure 15. Quantification of total phenolic compounds and DPPH scavenging capacity of *S. lycopersicum* L. extracts prepared in different conditions (A, B, C and D). Values presented are mean ± SD.

The use of 20 mL as extraction volume resulted in higher quantity (1.2 times) of phenolic compounds extracted (figure 15 B), while 10 mL allowed the extraction of 3.75 mg g^{-1} dry weight. Interestingly, the use of 40 mL lead to better results the in the DPPH assay, however, not higher enough than those values obtained with 20 mL and thus did not justify the use of the double amount of solvent for this type of extraction.

As expected, extracts prepared with mixtures of organic solvents and water (50% and 70% methanol) exhibited higher amount of phenolic compounds (Fig 15 C) [79]. Extracts prepared with 70% methanol had more phenolic compounds (1.1 times) and higher antiradicalar capacity (1.2 times) than extracts prepared with 50% methanol, and so this was the percentage chosen for future assays.

Although the time of extraction did not significantly affect the quantity of phenolic compounds extracted (Fig 15 D), exposure to ultrasounds for 60 minutes resulted in an extract with higher antiradicalar capacity (1.3 times), therefore this was considered the ideal time for extraction.

In brief, the chosen extraction conditions for the total phenolic compounds (TPC) quantification and Trolox equivalent antioxidant capacity (TEAC) by DPPH were: 20 mL of 70% methanol and exposure to ultrasounds for 60 minutes.

1.2. Identification of phenolic compounds by HPLC-DAD-MS^n

The tomato fruits extract obtained in the optimized conditions was analyzed by HPLC-DAD-MS^n in a tentative of identification of the main phenolic compounds. Several peaks were observed, and two main compounds were identified by comparing with standards: chlorogenic acid (peak 4) and rutin (peak 7) (Fig 16 and table 3).

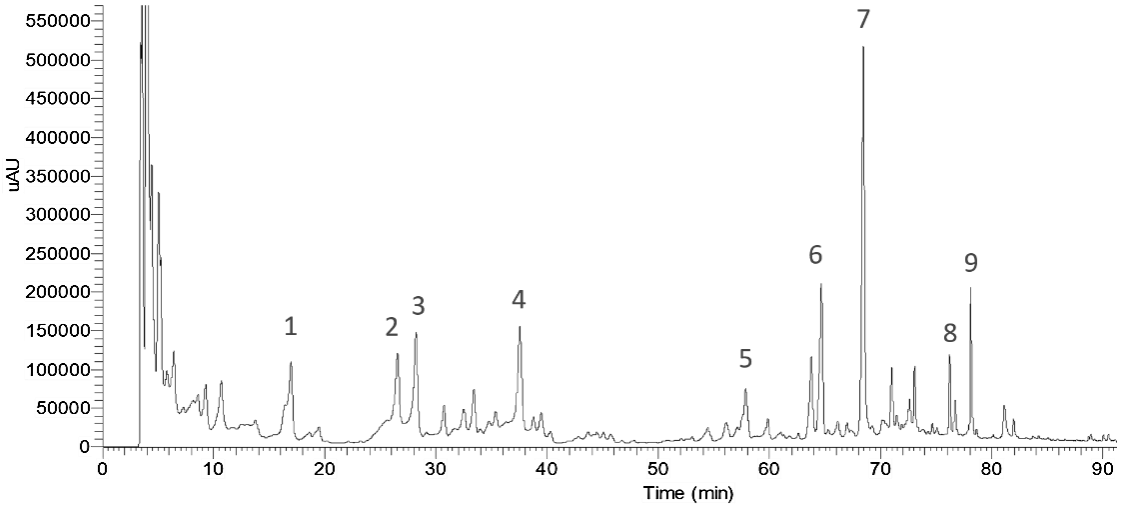


Figure 16. HPLC-DAD chromatogram at 280 nm of the methanolic extract of *S. lycopersicum*'s fruit extracts.

Table 3. Tentative identification of phenolic compounds in the methanolic extract of *S. lycopersicum*'s fruit extracts.

Peak	Retention time (min)	λ (nm)	$[M-H]^-$	MS^2 (*m/z*)	Tentative identification	Reference
1	17	271	565	323	unknown	-
2	27	295	325	163	Coumaric acid-O-hexoside	80
3	28	289, 316sh; 241	341	179	Caffeic-acid-O-hexoside	80
4	38	325, 296sh; 244	353	191	Chlorogenic acid	*
5	58	286	392	271	unknown	-
6	65	286	597	477, 387, 357	Phloretin-C-diglycoside	80
7	68	256; 355	609	301	Rutin	*
8	76	328, 296sh; 250	677	515	Tricaffeoylquinic acid	81
9	78	367, 382sh; 244	543	271	unknown	-

*reference standard

Peak 2 (*m/z* 325) was identified as a coumaric acid-O-hexoside. The ion with m/z 163 corresponds to a loss of a hexose (162) [80].

Peak 3 (*m/z* 341) presented the characteristic fragmentation of a caffeic acid-O-hexoside: the loss of a hexose (162) resulted in a fragment with *m/z* 179 [80].

Peak 6 (*m/z* 597) presented the fragmentation pattern expected for Phloretin-C-diglycoside [81,82].

Peak 8 (*m/z* 677) was identified as a tricaffeoylquinic acid; the loss of 162 corresponds to the loss of a caffeic acid unit and originates a dicaffeoylquinic acid (*m/z* 515) [80].

All these compounds have been already been identified in tomatoes [80,81].

Identification of peaks 1, 5 and 9 was not achieved because their molecular weight and fragmentation pattern did not correspond to the standards analyzed or described in the literature.

2. Effects of $CaCl_2$ treatments on tomato fruits

2.1. Morphological effects of $CaCl_2$ treatments

According to Arthur, Oduro and Patrick [10], $CaCl_2$ treatments would reduce decay and increase shelf life of tomato fruits. However, as visible in Table 3, treatment with 6% $CaCl_2$ had a negative effect on *S. lycopersicum* cv. Micro-Tom fruits, allowing the development of microorganisms, which did not happen in controls, or even with lower concentrations of $CaCl_2$, therefore these fruits were not used in subsequent analysis. There were no visible differences in morphology of tomatoes treated with 1% or 2% of $CaCl_2$ when compared with controls.

Table 4. *S. lycopersicum* cv. Micro-Tom fruits 1 or 2 weeks after application of $CaCl_2$ treatment

	1 week	2 weeks
control		
1%		
2%		
6%		

2.2. Effect of $CaCl_2$ on weight loss of tomato fruits

Tomato fruits weight loss increased significantly (between 1.5 and 1.9 times) with time of storage, regardless of the treatment. No statistically significant differences were found between weight loss of controls and fruits treated with $CaCl_2$ on both weeks (Fig 17).

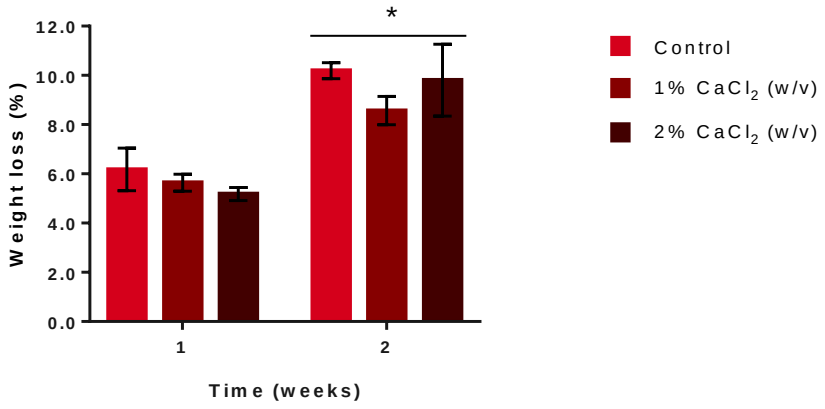


Figure 17. Weigh loss of *S. lycopersicum* cv. Micro-Tom fruits treated with different percentages of $CaCl_2$ after 1 or 2 weeks of storage. Values presented are mean ± SD. The bar indicates statistically significant differences between different times of storage.

2.3. Effect of $CaCl_2$ treatments on total phenolic compounds and antiradicalar capacity of tomato fruits

In both TPC and DPPH assays the results showed no statistically significant differences caused by both treatments with $CaCl_2$. However, there was a slightly increase in the amount of phenolic compounds after the 2% $CaCl_2$ treatment, which was more visible at 2 weeks (9.80 mg g^{-1} d. w. at week 1 and 11.5 mg g^{-1} d. w. at week 2). Antiradical capacity of fruits treated with 2% $CaCl_2$ (w/v) was also slightly higher (1.2 times) compared to fruits treated with 1% $CaCl_2$ (w/v) or controls (Fig 18).

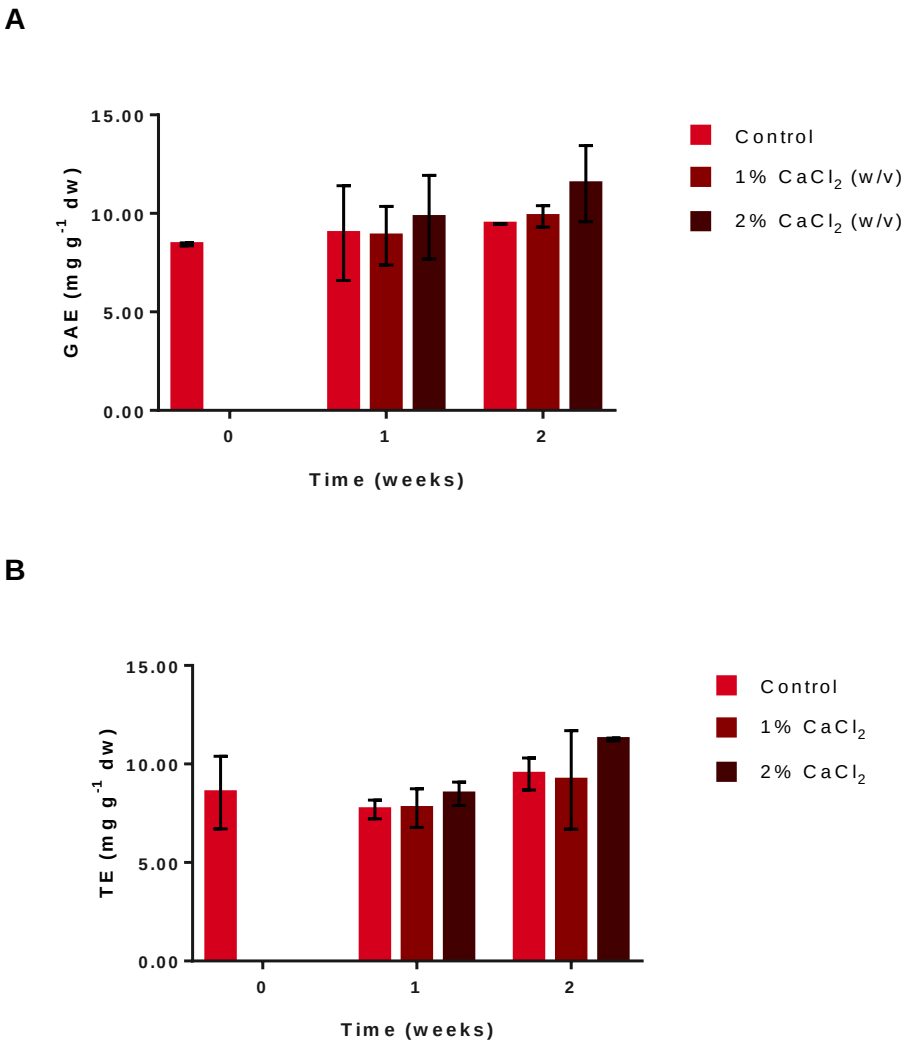


Figure 18. Quantification of total phenolic compounds (A) and DPPH scavenging capacity (B) of methanolic extracts of *S. lycopersicum* cv. Micro-Tom fruits treated with different percentages of $CaCl_2$ after 1 or 2 weeks of storage, and fruits without treatment at the time of harvest. Values presented are mean ± SD.

2.5. Identification of phenolic compounds by HPLC-DAD-MS after $CaCl_2$ treatments

S. lycopersicum cv. Micro-Tom extracts were analyzed by HPLC-DAD-MS in a tentative of identification of the main phenolic compounds and to evaluate the impact of calcium chloride on their concentration through 2 weeks of storage.

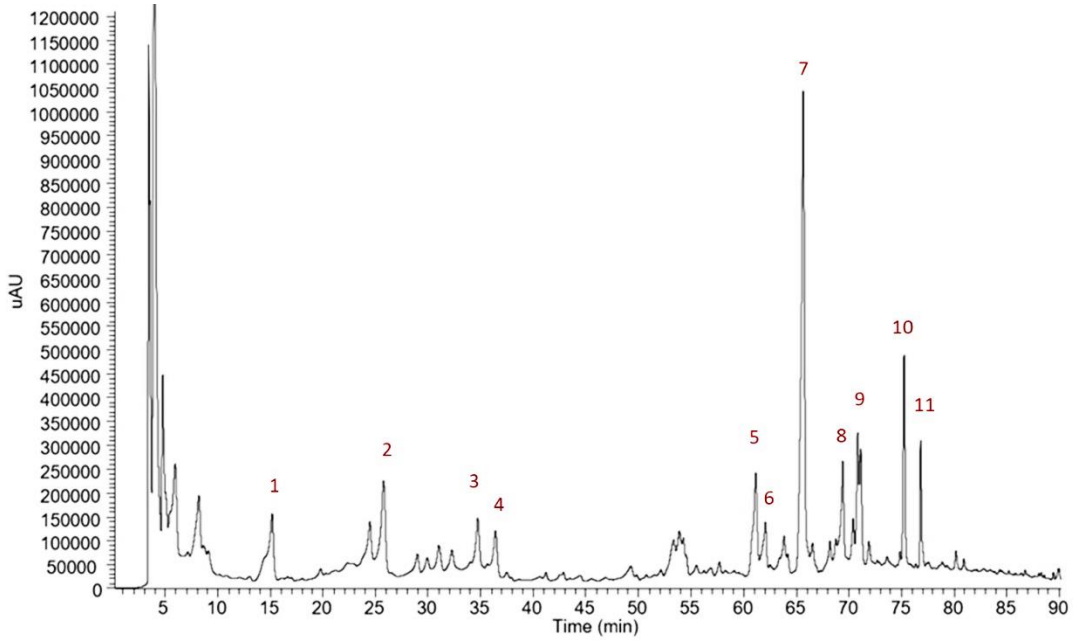


Figure 19. Typical HPLC-DAD chromatogram obtained at 280 nm of a methanolic extract of *S. lycopersicum* cv. Micro-Tom fruits treated with $CaCl_2$.

Peaks 3 (*m/z* 353), 4 (*m/z* 353) and 7 (*m/z* 609) were identified as two isomers of chlorogenic acid and rutin, respectively, by comparing with standards (Fig 19 and table 5).

Peak 2 (*m/z* 341) presented the characteristic fragmentation pattern of a caffeic acid-O-hexoside: the loss of a sugar originates the ion with *m/z* 179 and the loss of CO_2 originates the ion with *m/z* 135 [80].

Table 5. Tentative identification of phenolic compounds in methanolic extracts of *S. lycopersicum* cv. Micro-Tom fruits based on data obtain from HPLC-DAD-MS.

Peak	Retention time (min)	λ (nm)	$[M-H]^-$	MS^n (*m/z*)	Tentative identification	Reference
1	15	277	565	323, 211, 280	unknown	-
2	26	292	341	179, 135	Caffeic acid-O-hexoside	[80]
3	35	325, 310 sh	353	179, 191, 135	Chlorogenic acid	*
4	36	325, 310 sh	353	191, 179, 173	Chlorogenic acid	*
5	61	283, 334	433		unknown	-
6	62	296	597	477, 357, 387	Phloretin-C-diglycoside	[81]
7	66	352	609	301, 179, 271	Rutin	*
8	69	331, 310 sh	515	353, 179, 173, 191	Dicaffeoylquinic acid	[80]
9	71	343, 295	593	285, 257	Kaempferol rutinoside	[35]
10	75	295, 328	677	515, 353	Tricaffeoylquinic acid	[81]
11	77	361, 382	271	151, 107	Naringenin	[83]

*reference standard

Peak 6 (*m/z* 597) was identified as a phloretin-C-diglycoside. Ions with *m/z* 477, 357 and 387 (difference of 90 and 120 mu) result from sugar losses [81].

Peak 8 (*m/z* 515) was identified as a dicaffeoylquinic acid. The ion with *m/z* 353 resulted from the loss of a caffeic acid unit, and the ion with *m/z* 191 corresponds to the deprotonated quinic acid [80].

Peak 9 (*m/z* 593) corresponds to a kaempferol rutinoside, according to Barros, Duenas, Pinela, Carvalho, Buelga and Ferreira [35].

Peak 10 (*m/z* 677) presents the typical fragmentation pattern of a tricaffeoylquinic acid and had already been identified in the tomato fruits used in the extract optimization process [81].

Peak 11 (*m/z* 271) corresponds to naringenin, one of the major polyphenols of tomatoes [80,83].

2.5.1. Effect of $CaCl_2$ treatments on the concentrations of individual compounds

The concentration of phenolic compounds separated by HPLC was calculated for all conditions in order to evaluate if the calcium chloride would have any impact on the concentration of the extracted phenolic compounds. Values obtained are summarized in table 5. In order to better interpret these results, and for the most relevant compounds, concentration values in mg/g d.w. were converted to a percentage of concentration of the same compound in fruits without treatment and at harvest (Fig 20).

Table 6. Concentration of individual phenolic compounds identified on methanolic extracts of *S. lycopersicum* cv. Micro-Tom fruits at harvest and after 1 and 2 weeks of storage after the respective $CaCl_2$ treatments. Results are expressed in gallic acid equivalents (GAE) mg/g dry weight ± SD.

		1 week			2 weeks		
Peak	**Control**	**Control**	**1% $CaCl_2$**	**2% $CaCl_2$**	**Control**	**1% $CaCl_2$**	**2% $CaCl_2$**
1	8.2 ± 1.7	9.4 ± 0.9	16.9 ± 9.2	26.8 ± 6.1	26.7 ± 15.4	20.2 ± 9.8	24.2 ± 15.5
2	18.3 ± 3.7	24.6 ± 0.3	20.1 ± 9.2	23.5 ± 7.8	18.4 ± 2.6	24.9 ± 13.5	17.9 ± 1.0
3	25.7 ± 14.0	19.3 ± 3.5	16.0 ± 9.6	19.4 ± 9.3	14.0 ± 4.4	18.8 ± 11.9	13.0 ± 5.0
4	9.5 ± 1.5	10.7 ± 0.7	8.4 ± 0.5	10.5 ± 1.0	6.5 ± 1.9	7.8 ± 0.4	12.9 ± 6.4
5	19.5 ± 5.0	22.9 ± 5.8	19.0 ± 0.6	28.9 ± 3.5	23.8 ± 5.7	20.0 ± 1.8	39.0 ± 7.2
6	9.8 ± 1.2	7.4 ± 1.5	9.4 ± 0.1	9.4 ± 1.4	9.2 ± 0.3	10.3 ± 0.1	11.4 ± 1.0
7	81.4 ± 0.4	77.9 ± 8.0	80.3 ± 9.4	101.6 ± 26.9	116.2 ± 27.3	98.1 ± 39.9	103.0 ± 12.3
8	14.9 ± 6.5	12.8 ± 5.5	14.8 ± 13.0	20.1 ± 4.4	6.2 ± 1.3	14.3 ± 3.5	19.1 ± 3.1
9	15.7 ± 6.8	14.5 ± 10.2	15.8 ± 9.6	10.1 ± 2.8	26.5 ± 17.9	17.3 ± 12.5	12.5 ± 6.6
10	17.7 ± 7.2	20.2 ± 4.0	19.0 ± 13.9	24.1 ± 4.8	13.4 ± 7.8	22.5 ± 6.9	29.8 ± 13.6
11	21.5 ± 6.1	13.1 ± 0.3	13.4 ± 4.0	15.5 ±0.4	8.7 ± 3.1	9.1 ± 2.8	8.9 ± 0.6

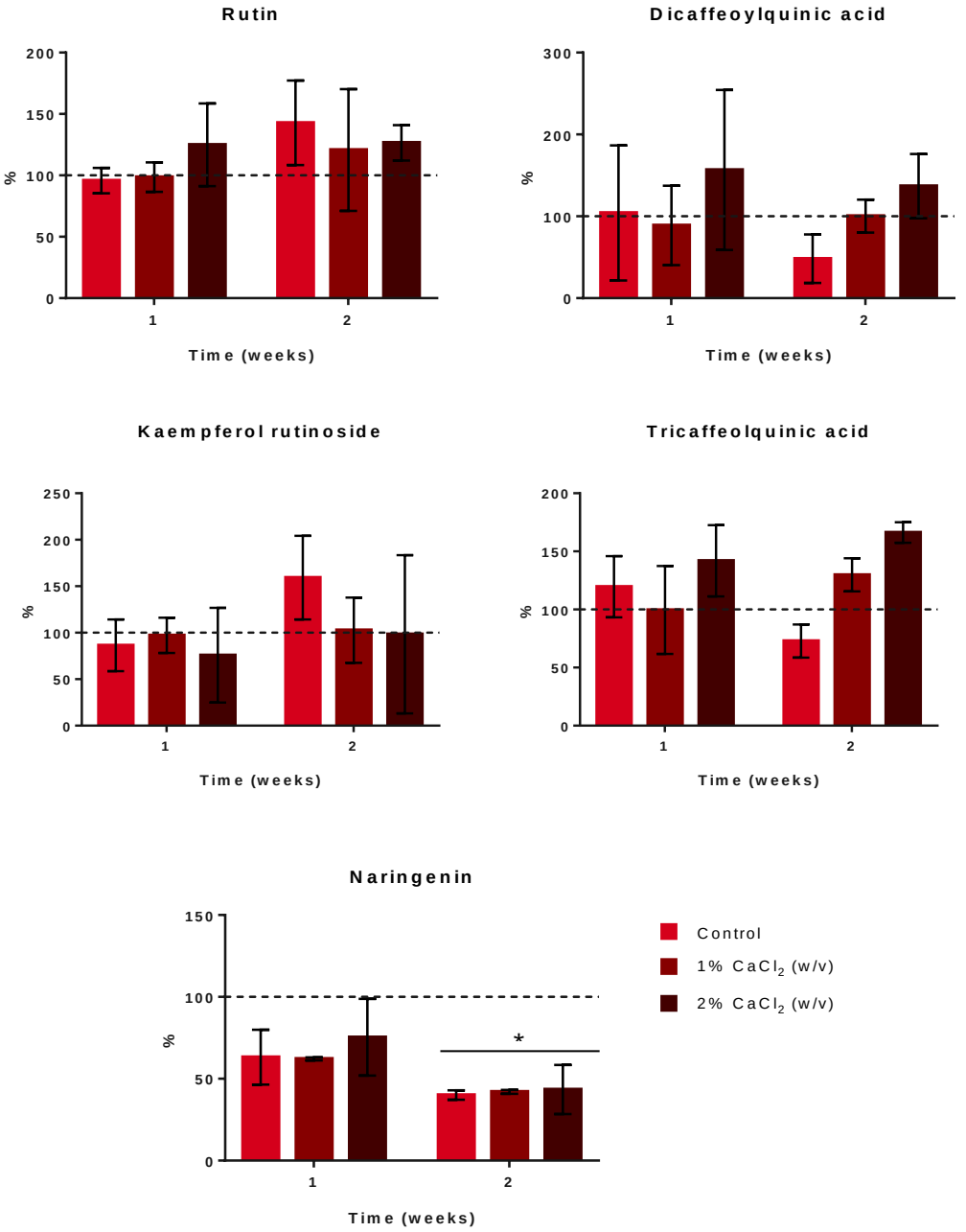


Figure 20. Concentration of individual phenolic compounds identified on methanolic extracts of *S. lycopersicum* cv. Micro-Tom fruits treated with different percentages of $CaCl_2$ after 1 or 2 weeks of storage compared to fruits without treatment at harvest. Values presented are mean ± SD. * indicates statistically significant differences from control, within the same week. The bar indicates statistically significant differences between different times of storage.

Kaempferol rutinoside and rutin exhibited no statistically significant differences during the two weeks analyzed, both for 1% and 2% $CaCl_2$ treatments. Dicaffeoylquinic acid showed an increase in its concentration on week 2 with the increase of $CaCl_2$

concentration, however it was not statistically significant. For these 3 compounds, the concentrations in all conditions was close to the ones at harvesting-time (100%). Tricaffeoylquinic acid showed a similar response to treatments to that of dicaffeoylquinic acid, however, at week 2, treatment with 2% $CaCl_2$ (w/v) lead to an increase in its concentration that was statistically significant. At week 2, tricaffeoylquinic acid concentration was 2.3 times higher for 2% $CaCl_2$ (w/v) treatment than in control. Naringenin concentration was not affected by $CaCl_2$ treatments but decreased with time (37% on average), in a statistically significant manner.

2.6. Online HPLC-DPPH analysis

DPPH-Online method was used to find out which phenolic compounds were contributing to antioxidant capacity of *S. lycopersicum* cv. Micro-Tom fruits (Fig 21 and table 7).

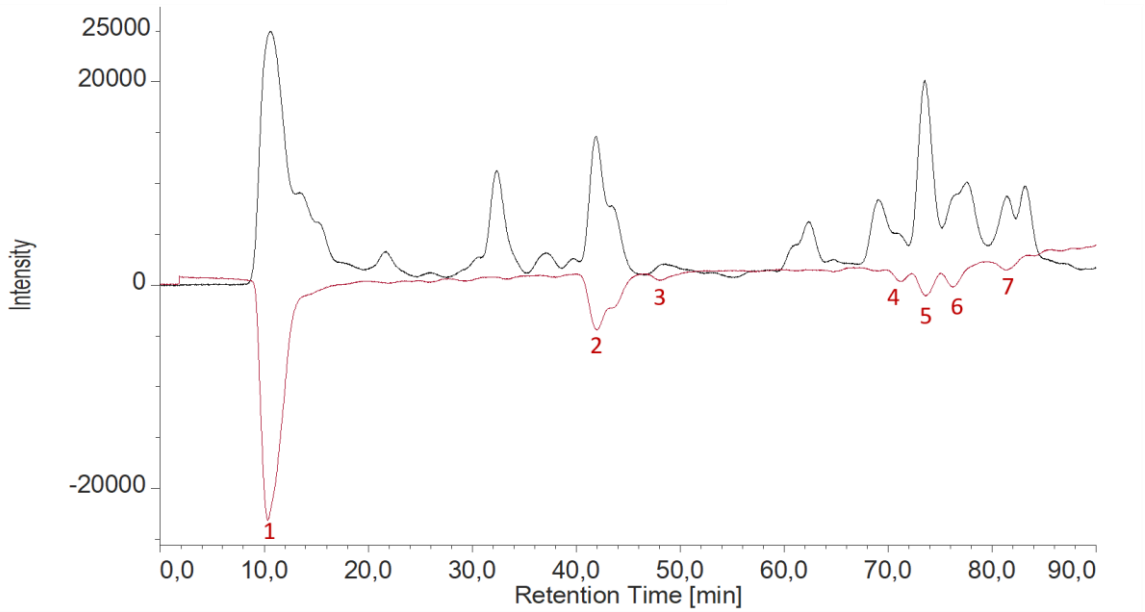


Figure 21. Typical HPLC-DAD chromatogram obtained for methanolic extracts of *S. lycopersicum* cv. Micro-Tom fruits without $CaCl_2$ treatment at harvesting time. Chromatogram in black was obtained at 280 nm and chromatogram in dark red at 515 nm.

The group of compounds eluted at 10 minutes was responsible for the most antioxidant capacity of the extract analyzed. These are the compounds eluted at the

beginning of the run (compounds with higher polarity), and the HPLC conditions used were not adequate for their separation, therefore it was not possible to identify them.

Table 7. Tentative identification of phenolic compounds with antiradicalar capacity in methanolic extracts of *S. lycopersicum* cv. Micro-Tom fruits at harvest based on data obtain from DPPH-Online.

Peak	Retention Time	Area	%	Identification
1	10	3310644	70.3	unknown
2	42	854628	18.2	Chlorogenic acid
3	48	70142	1.5	unknown
4	71	66598	1.4	unknown
5	74	185749	3.9	Rutin
6	76	99906	2.1	Dicaffeoylquinic acid
7	81	119061	2.5	Tricaffeoylquinic acid

From the identified compounds, chlorogenic acid was the one with higher antiradicalar capacity (18.2%). The other major contributors, although with much lower antiradicalar capacity were, respectively, rutin, tricaffeoylquinic and diccaffeoylquinic acids.

2.7. Effect of $CaCl_2$ on Glutathione levels

2.7.1. [GSH] and [GSSG]

GSH levels increased during the 2 weeks of storage but such increase was not enough for it to be considered statistically significant. None of the $CaCl_2$ treatments affected GSH concentration during the two weeks of storage (Fig 22).

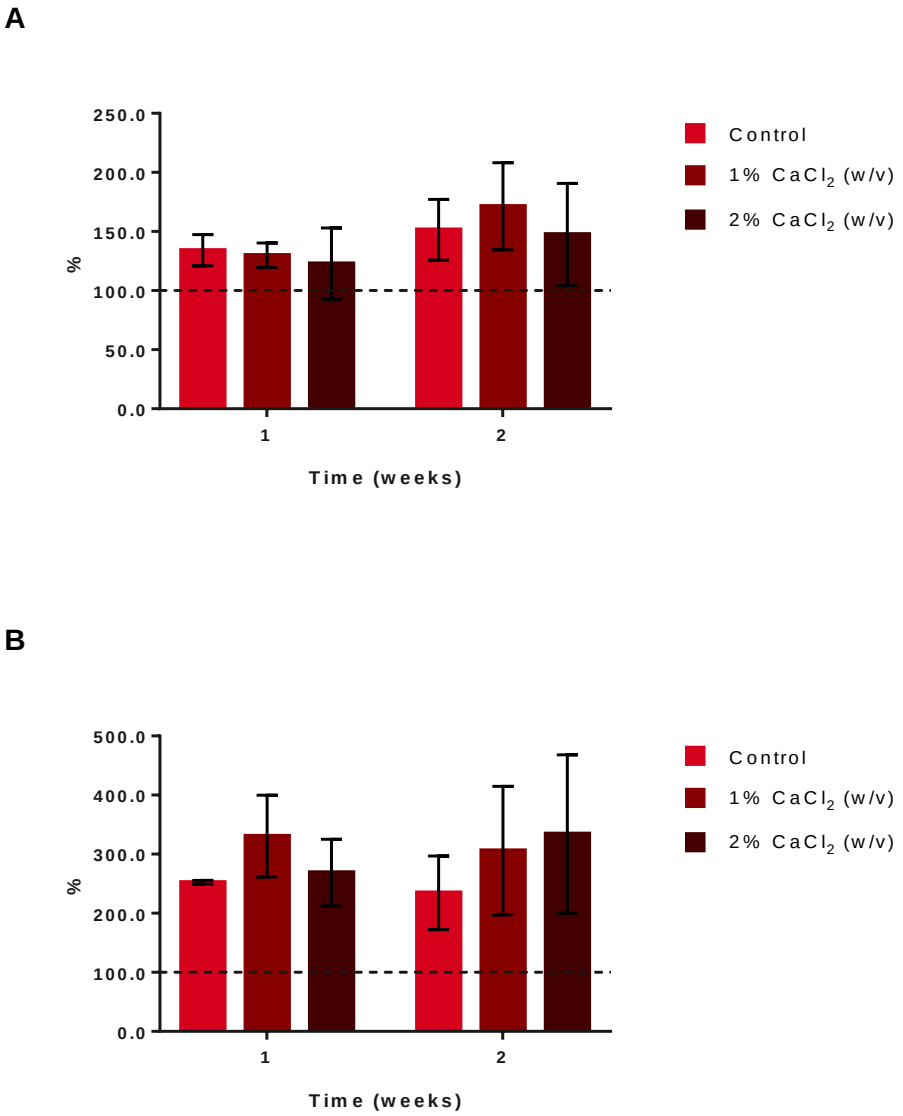


Figure 22. Reduced (A) and oxidized (B) glutathione levels in *S. lycopersicum* cv. Micro-Tom fruits treated with different percentages of $CaCl_2$ after 1 or 2 weeks of storage compared to fruits without treatment at harvest. Values presented are mean ± SD.

GSSG levels did not vary significantly between the first and second week. At week 2 there was an increase in GSSG levels with the increase in $CaCl_2$ concentration that was not statistically significant. It is important to note that on both weeks GSSG values were much larger (between 2 and 3 times higher) than GSSG levels of fruits at harvest.

2.7.2. GSH/GSSG

After quantification of GSH and GSSG, the ratio GSH/GSSG, an indicator of the redox state of the fruits was determined [37]. GSH/GSSG values were below 100% in all conditions or, in another words, in all situations the values were lower than those of fruits at harvest (100%) (Fig 23).

In week 1, GSH/GSSG ratio was close to 50% in all situation, which means it was nearly half of GSH/GSSG ratio of fruits at harvest, and it is not evident a dose-dependent change in this ratio in response to $CaCl_2$. On week 2, the GSH/GSSG ratio decreases with higher $CaCl_2$ concentrations, with values ranging from 68% for control and 45% for treatment with 2% $CaCl_2$ (w/v).

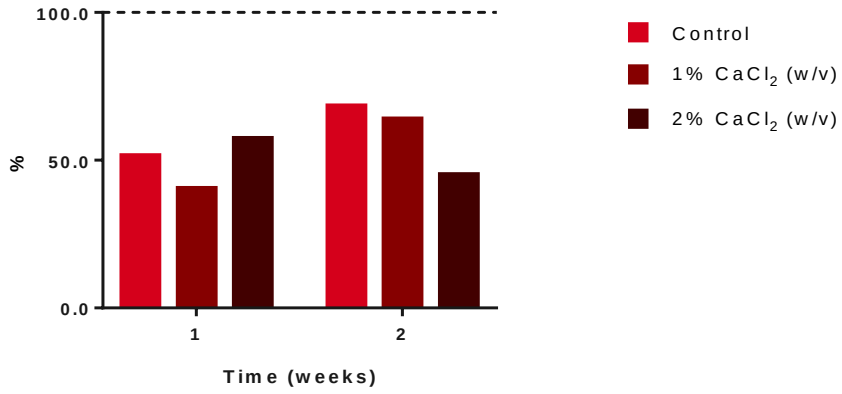


Figure 23. Reduced/Oxidized glutathione ratio in *S. lycopersicum* cv. Micro-Tom fruits treated with different percentages of $CaCl_2$ after 1 or 2 weeks of storage, compared to fruits without treatment at harvest. Values presented are mean.

2.8. Effect of $CaCl_2$ on enzymatic activity

2.8.1. Glutathione reductase

Activity of glutathione reductase did not suffer any statistical significantly variation during the weeks analyzed (Fig 24).

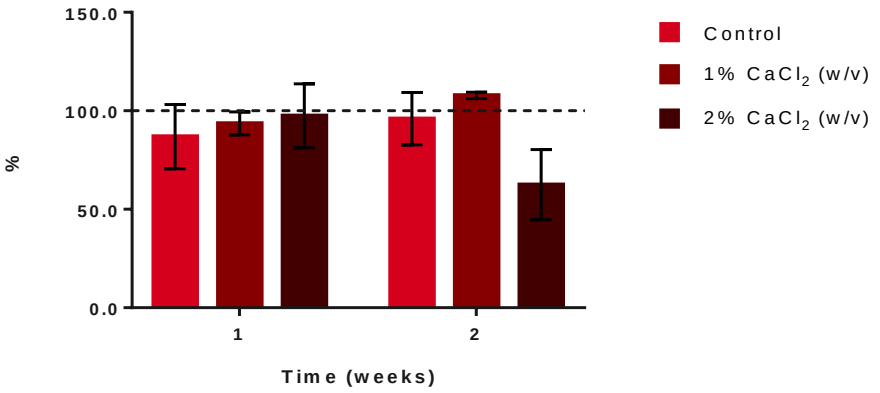


Figure 24. Glutathione reductase activity in *S. lycopersicum* cv. Micro-Tom fruits treated with different percentages of $CaCl_2$ after 1 or 2 weeks of storage compared to fruits without treatment at harvest. Values presented are mean ± SD.

Curiously, after one week, the increase in the $CaCl_2$ concentration caused a minor increase on GR activity, on a concentration-dependent manner. On week 2, treatment with 2% $CaCl_2$ (w/v) induced a decrease in GR activity (GR activity in these fruits was 62,5% of fruits at harvest time), although with no statistical significance. Nevertheless, in most situations, GR activity values resembled those of fruits at harvest time, without any treatment.

2.8.2. γ-ECS

In week 1 there was not a dose-dependent variation of γ-ECS activity in response to $CaCl_2$: treatment with 1% $CaCl_2$ resulted in a statistically significant increase in γ-ECS activity (3.4 times higher than control), but the increase induced by 2% $CaCl_2$ treatment (2.4 times higher than control) was not high enough to be significant (Fig 25).

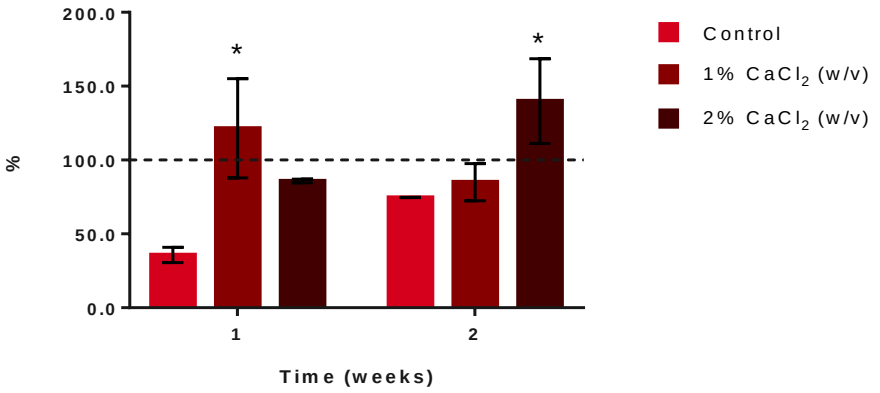


Figure 25. γ-ECS activity in *S. lycopersicum* cv. Micro-Tom fruits treated with different percentages of $CaCl_2$ after 1 or 2 weeks of storage compared to fruits without treatment at harvest. Values presented are mean ± SD. * indicates statistically significant differences from control, within the same week.

On the other hand, on week 2 there was an increase in γ-ECS activity with both $CaCl_2$ concentrations, being only significant for the 2% (w/v) $CaCl_2$ treatment (1.9 times higher than control). On both weeks, controls had lower γ-ECS activity than fruits at harvest.

2.8.3. Glutamine synthetase

Both on weeks 1 and 2 the increase of $CaCl_2$ concentration lead to a slight increase of the tomato fruits' GS activity, although with no statistical significance in both weeks 1 and 2 (Fig 26).

No statistically significant differences in GS activity between week 1 and week 2 of storage time were found. In all conditions GS activity values were similar to the ones of fruits at harvest, without treatment.

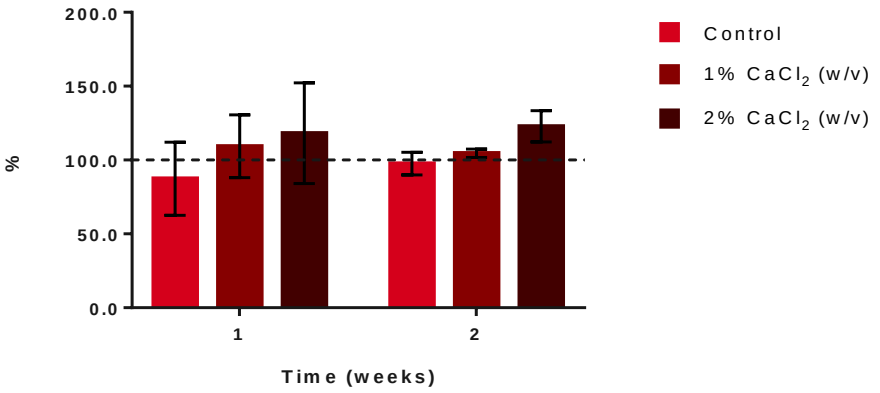


Figure 26. Glutamine synthetase activity in *S. lycopersicum* cv. Micro-Tom fruits treated with different percentages of $CaCl_2$ after 1 or 2 weeks of storage compared to fruits without treatment at harvest. Values presented are mean ± SD.

2.9. Effect of $CaCl_2$ on protein content

On both weeks, treatments with 1% and 2% (w/v) $CaCl_2$ caused, on average, 2 times more protein than the respective controls (statistically significant differences) (Fig 27). No statistically significant differences between the 2 weeks monitored were found.

Unlike in the treated groups, the amount of protein in controls decreased with time. Protein content of controls at both weeks was lower than protein content of fruits at harvest, while in treated fruits the protein content remained similar to the initial value or even slightly increased.

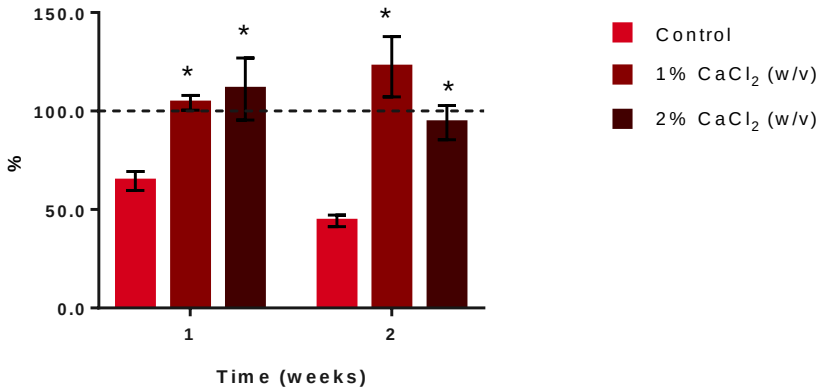


Figure 27. Protein quantification in *S. lycopersicum* cv. Micro-Tom fruits treated with different percentages of $CaCl_2$ after 1 or 2 weeks of storage compared to fruits without treatment at harvest. Values presented are mean ± SD. * indicates statistically significant differences from control, within the same week.

Discussion

Calcium chloride treatments did not affect morphological aspects of *S. lycopersicum* cv. Micro-Tom fruits

Even though previous studies reported a positive effect of $CaCl_2$ on reducing decay and weight loss of treated fruits [10,54-56], such effect was not visible in this study. As a matter of fact, treatments with 1% and 2% $CaCl_2$ (w/v) did not cause any visible effect on color or texture of tomatoes. Besides, when tomato fruits were treated with 6% $CaCl_2$ (w/v) there was a development of microorganism at the fruits' surface, a result not expected considering $CaCl_2$ is reported to have antimicrobial activity [63]. Regarding fruits' weight, treatments did not reduce weight loss during storage, as expected [10,54-56]. Although not statistically significant, the minor decrease in weight loss in treated fruits suggests $CaCl_2$ may have a positive effect in higher concentrations (but lower than 6%). The discrepancy between results obtained and expected may be due to the use of different cultivars and different storage conditions than in the previous studies [10,54-56].

To clarify the effect $CaCl_2$ may have on tomato fruits, this treatment could be tested with more samples, during an extended time, and further concentrations in the range of 2-6%.

Calcium chloride treatments exhibited a limited effect on phenolic compounds and antiradicalar capacity of *S. lycopersicum* cv. Micro-Tom fruits' extracts

TPC and DPPH assays showed similar results, indicating that variations on the total of phenolic compounds are reflected in antiradicalar capacity of tomato fruits extracts.

With time, phenolic content did not change significantly, however, there was a tendency for an increase caused by $CaCl_2$ treatments. When calcium bonds with cellular wall components, a stabilization of cellular structure may occur that will reduce the release of phenolic compounds, which may justify the obtained results [62].

Regarding individual compounds, rutin was clearly the one with higher concentrations during the two weeks of storage. According to the literature, in normal-sized tomatoes, the main phenolic compounds present are chlorogenic acid, quercetin,

naringenin and rutin [7,16,35,84]. Chemical composition is dependent of genotype and environmental factors, and phenolic compounds can be used to distinguish between different varieties or cultivars. In addition, the absence of quercetin among the compounds identified cannot be considered a characteristic of this variety because it has already been identified in *S. lycopersicum* cv Micro-Tom [1,7,8,31-34].

Even though $CaCl_2$ treatments induced an increase in concentrations of some of the identified phenolic compounds, particularly tricaffeoylquinic acid (its concentration more than doubled in the second week, with application of 2% $CaCl_2$ (w/v)), most of these changes were not significant. If added to this the fact that concentration of some compounds tended to decrease with higher $CaCl_2$ concentrations, it is possible to say that the results obtained by HPLC-DAD-MS are concordant with the absence of significant variations in total phenolic compounds observed in TPC assay.

Analyzing HPLC-DAD-MS and DPPH-Online results together, it was observed that chlorogenic acid, the isolated compound with higher antiradicalar capacity, did not follow any specific pattern in response to $CaCl_2$ treatments, although some authors reported a decrease in chlorogenic acid concentration during the ripening process [84,85]. Additionally, rutin, the second most abundant antiradicalar compound, did not have its concentration altered by the application of $CaCl_2$, a result already observed in other varieties [85-88]. Therefore, $CaCl_2$ treatments did not have a significant impact on phenolic compounds concentration and, consequently, on antiradicalar capacity of *S. lycopersicum* cv. Micro-Tom fruits.

Calcium chloride treatments lowered the redox state in *S. lycopersicum* cv. Micro-Tom fruits

GSH/GSSG ratio is an indicator of the redox state of the fruits, therefore it is important to understand how and why it changes in response to post-harvest calcium chloride treatments.

At the end of the first week, the values of GSH/GSSG ratio were nearly half of those of fruits at harvesting time, in all situations, and a response to $CaCl_2$ in function of its concentration was not observed. At week 2, GSH/GSSG ratio decreased in response to higher $CaCl_2$ concentrations. To understand why this happened, the impact of $CaCl_2$ on enzymes involved in synthesis and recycling of glutathione, and how that affected GSH and GSSG levels, was further analyzed.

Although not statistically significant, the treatment with 2% $CaCl_2$ induced a decrease in GR activity on week 2, which can explain the increase observed in GSSG concentration during the same week.

Looking at the enzymes responsible for GSH synthesis, at week 2, $CaCl_2$ treatments caused an increase in γ-ECS activity, which means that there was an increase in GSH synthesis. Such increase was only possible because GS activity on week 2 was similar to its activity at harvest, although tending to increase with the $CaCl_2$ treatments, meaning there were amino acids available for γ-ECS to use for GSH synthesis. In spite of a diminished GR activity, meaning there was less GSSG being converted to GSH, the increase in GSH synthesis was enough for GSH concentration to be slightly higher on week 2 than on week 1.

The combining activity of these 3 enzymes (GR, GS and γ-ECS) resulted in a lower GSH/GSSH ratio on treated fruits, since the increase in GSH synthesis was not enough to offset higher GSSG concentrations.

To conclude, $CaCl_2$ had a negative effect on the redox state of tomato fruits, lowering the GSH/GSSG ratio.

Calcium chloride treatments prevented protein degradation in *S. lycopersicum* cv. Micro-Tom fruits

Protein content was the parameter exhibiting a more evident response to calcium chloride treatments. On control groups, protein content decreased with time during the two weeks of storage. Meanwhile, protein content on treated fruits remained similar to the protein content of fruits at harvesting time, and was significantly higher than in controls with the same storage times. Therefore, $CaCl_2$ prevented the natural degradation of proteins with time. There is not much information in the literature about protein levels in fresh tomatoes during ripening and/or storage, since most studies focused on the analysis of antioxidant levels (such as carotenoids and phenolic compounds) and sugar content. According to Raffo, et al. [89], protein content in tomato fruits does not change during ripening, however, it is important to notice that his team studied the ripening of the tomatoes on the vine, while in this study, the change in protein levels was analyzed after the harvesting of the fruits for two weeks. Regarding protein content per gram of fresh weight, Raffo et al. [89] reported levels between 1.0 and 1.3 g of

protein/100 g f.w.. At harvest, Micro-Tom tomatoes had 3.7 g of protein/100 g f.w., but at the end of two weeks of storage it decreased to 1.6 g/100 g f.w, a value similar to the one described by Raffo and his team, while fruits treated with $CaCl_2$ maintained the protein content in the range 3.5 to 4.6 g /100 g f.w. during the two weeks of storage.

Concluding remarks

Treatments with 1% and 2% $CaCl_2$ (w/v) did not cause any visible effect on color or texture of tomatoes. In tomato fruits treated with 6% $CaCl_2$ (w/v) a development of microorganism at the fruits' surface was observed, therefore these fruits were not used in subsequent analysis. Besides, a reduce in weight loss during storage of treated fruits was not perceived, unlike expected

Variations on the total of phenolic compounds were not reflected in antiradicalar capacity of tomato fruits extracts. Neither phenolic content nor antioxidant capacity did change significantly with $CaCl_2$ treatments.

Regarding phenolic profile of tomato fruits, rutin was clearly the one in higher concentrations during the two weeks of storage and its concentration was not affected by $CaCl_2$ treatments. Chlorogenic acid, the isolated compound with higher antiradicalar capacity, did not follow any specific pattern in response to treatments. Tricaffeoylquinic acid concentration significantly increased with application of 2% $CaCl_2$ (w/v) on week 2. Although already identified in *S. lycopersicum* cv Micro-Tom fruits, quercetin, one of the main flavonoids usually found in tomatoes, was not identified neither in controls nor in treated fruits.

2% $CaCl_2$ (w/v) treatment induced an increase in GSH synthesis by increasing GS and γ-ECS activity, however, it also caused a decrease in GR activity, meaning there was less GSSG recycled to GSH. The combined activity of these three enzymes (GR, GS and γ-ECS) resulted in a lower GSH/GSSG ratio on treated fruits because the increase in GSH synthesis was not enough to offset the higher GSSG concentrations.

During the two weeks of storage $CaCl_2$ prevented the natural degradation of proteins with time, resulting in a significantly higher protein content on treated fruits than in controls.

In conclusion, it is possible to state that treatment with calcium chloride to prolong shelf life of tomatoes is an advantageous procedure as it did not did not affect morphological aspects of the fruits as well as did not significantly alter their organoleptic characteristics, such as phenolic compounds and antiradicalar capacity. Furthermore, it also prevented protein breakdown, contributing to the maintenance of this nutritional aspect of the fruits.

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