

**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Emerging technologies to enhance starch performance: ozone,
ultrasound and irradiation**

Nanci Castanha da Silva

Thesis presented to obtain the degree of Doctor in
Science. Area: Food Science and Technology

**Piracicaba
2019**

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Food Engineer

Emerging technologies to enhance starch performance: ozone, ultrasound and irradiation

Advisor:
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Dedico aos meus pais, Zé e Lia.
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EPIGRAPH

"The important thing is not to stop questioning".

Albert Einstein

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RESUMO

Uso de tecnologias emergentes para melhoria do desempenho de amidos: ozônio, ultrassom e irradiação

Amidos são largamente utilizados em diversos produtos e processos industriais. Cada aplicação, porém, demanda diferentes funcionalidades, as quais muitas vezes não são apresentadas por amidos nativos de fontes comerciais. Para contornar esta questão, duas abordagens podem ser utilizadas: (1) explorar amidos não-modificados, especialmente de fontes não convencionais e (2) explorar diferentes técnicas de modificação de amidos. Na presente Tese, ambas as abordagens foram exploradas, com o objetivo principal de obter amidos com melhor desempenho para diferentes aplicações. Na primeira parte da Tese, focada no uso de amidos em sua forma nativa, além da caracterização de uma fonte botânica não convencional de amido (raiz de arracacha), explorou-se também o processamento de amidos sob condições não-padrão. Em relação ao amido de arracacha, as propriedades funcionais obtidas foram muito promissoras do ponto de vista industrial. Ainda, ao se alterar as condições de processamento, demonstrou-se que é possível melhorar o desempenho de amidos nativos, sem a necessidade de modificá-los. Porém, apesar de trazer um apelo “natural” para alimentos processados, o uso de amidos nativos ainda pode ser limitado para algumas aplicações. Portanto, na segunda parte da Tese, explorou-se três diferentes tecnologias emergentes para modificação de amido: ozônio, ultrassom e irradiação. Estas técnicas foram selecionadas por apresentarem algumas vantagens em relação às tradicionalmente empregadas na indústria, tais como: serem mais eficientes, mais seguras e/ou ambientalmente mais amigáveis. Dentre as tecnologias exploradas, o ozônio provou ser versátil e bastante eficiente nas condições avaliadas. Cada condição de ozonização utilizada, combinada com diferentes fontes de amido, resultou em propriedades funcionais distintas, aumentando as possibilidades de aplicação dos amidos avaliados. Por outro lado, a tecnologia de ultrassom só apresentou efeitos mensuráveis quando combinada com a tecnologia de ozônio, nas condições aqui avaliadas. Por fim, considerando a irradiação, a mesma provou ser um processo eficiente e altamente penetrante, resultando em propriedades com diferentes possibilidades de aplicação para os amidos. Em todos os casos, discussões mecânicas foram realizadas de forma a correlacionar a estrutura dos amidos, sua modificação nas diferentes condições de processo, as propriedades obtidas e possíveis aplicações. Como conclusão, destacamos que o uso de técnicas de modificação traz uma gama maior de aplicações para diferentes fontes de amido. No entanto, as particularidades e limitações de cada processo de modificação precisam ser consideradas antes de sua seleção.

Palavras-chave: 1. Amido 2. Propriedades 3. Tecnologias emergentes 4. Ozônio 5. Ultrassom 6. Irradiação

ABSTRACT

Emerging technologies to enhance starch performance: ozone, ultrasound and irradiation

Starches are widely used in several industrial products and processes. Each application, however, requires different functionalities, which may be not presented by native starches from commercial sources. To work around this issue, two approaches can be used: (1) exploring non-modified starches, especially from unconventional sources and (2) exploring different starch modification techniques. In the present Thesis, both approaches were investigated, with the main objective of enhancing the performance of starches for different applications. In the first part of the Thesis, focused on the use of starches in their native form, besides the characterization of a unconventional botanical source of starch (arracacha root), the processing of starches under non-standard conditions was also explored. In relation to the arracacha starch, the obtained functional properties were very promising from an industrial point of view. Also, by changing the processing conditions, it was shown that it is possible to improve the performance of native starches without the need of modifying them. However, despite bringing a “natural” claim to processed foods, the use of native starches may still be limited for some applications. Therefore, in the second part of the Thesis, three different emerging technologies for starch modification were explored: ozone, ultrasound and irradiation. These techniques were selected because they have some advantages over those traditionally used in the industry, such as being more efficient, safer and/or more environmentally friendly. Among the technologies here explored, ozone proved to be a versatile and efficient technology at the evaluated conditions. Each ozonation condition, combined with different starch sources, resulted in distinct functional properties, boosting the possibilities of application for the analysed starches. On the other hand, the ultrasound technology only presented measurable effects when combined with the ozone technology, at the conditions here evaluated. Finally, considering the irradiation technology, it proved to be an efficient and highly penetrating process, resulting in different possibilities of application for the evaluated starches. In all cases, mechanistic discussions were performed in order to correlate the starch structure, its modification using different processes conditions, the obtained properties and possible applications. In conclusion, we highlight that the use of modification processes brings a broader range of applications for starches. However, the particularities and limitations of each modification process need to be considered before its selection.

Keywords: 1. Starch 2. Properties 3. Emerging technologies 4. Ozone 5. Ultrasound 6. Irradiation

RESUMEN

Tecnologías emergentes para mejorar la funcionalidad del almidón: ozono, ultrasonido e irradiación

El almidón es ampliamente utilizado en diversos productos y procesos industriales. Sin embargo, cada aplicación requiere diferentes funcionalidades, que a menudo no se encuentran en los almidones nativos de fuentes comerciales. Para resolver este problema, se pueden usar dos enfoques: (1) explorar almidones no modificados, especialmente de fuentes no convencionales y (2) explorar diferentes técnicas de modificación de almidón. En la presente Tesis, fueron investigados ambos enfoques, con el objetivo principal de obtener almidones con mejor funcionalidad para diferentes aplicaciones. En la primera parte de la Tesis, centrada en el uso de almidón en su forma nativa, además de la caracterización de una fuente botánica no convencional de almidón (raíz de arracacha), también se exploró el procesamiento de almidón en condiciones no estándar. Considerando el almidón de arracacha, las propiedades funcionales obtenidas fueron muy prometedoras desde un punto de vista industrial. Además, al cambiar las condiciones de procesamiento, se demostró que es posible mejorar la funcionalidad de los almidones nativos sin la necesidad de modificarlos. Sin embargo, a pesar de ser considerado como compuesto más "natural" para los alimentos procesados, su uso puede ser limitado para algunas aplicaciones. Por lo tanto, en la segunda parte de la Tesis, se exploraron tres tecnologías emergentes para la modificación del almidón: ozono, ultrasonido e irradiación. Estas técnicas se seleccionaron porque tienen algunas ventajas sobre las utilizadas tradicionalmente en la industria, como ser más eficientes, más seguras y/o más ecológicas. Entre las tecnologías explotadas, el ozono demostró ser versátil y bastante eficiente en las condiciones evaluadas. Además, cada tiempo de ozonización evaluado, combinado con diferentes fuentes de almidón, dio como resultado propiedades funcionales distintas, aumentando las posibilidades de aplicación de los almidones analizados. Por otro lado, en las condiciones aquí evaluadas, la tecnología de ultrasonido solo presentó efectos medibles cuando se combinó con la tecnología de ozono. Finalmente, la tecnología de irradiación resultó ser un proceso eficiente y altamente penetrante, que resultó en propiedades aplicables para las muestras de almidón. En todos los casos, se realizaron discusiones mecanicistas para correlacionar la estructura del almidón, su modificación en las diferentes condiciones del proceso, las propiedades obtenidas y las posibles aplicaciones. En conclusión, destacamos que el uso de muestras de almidón modificado trae una mayor gama de aplicaciones. Sin embargo, las particularidades y limitaciones de cada proceso de modificación deben ser consideradas antes de su selección.

Palabras clave: 1. Almidón 2. Propiedades 3. Tecnologías emergentes 4. Ozono 5. Ultrasonido 6. Irradiación

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1. INTRODUCTION AND THESIS ORGANIZATION

Starches are biopolymers found in nature, being abundant in several botanical sources, and their importance is undeniable from nutrition to industrial application. In fact, starches are valuable ingredients used in different industries, such as food, feed, chemical, petrochemical, pharmaceutical, textile, paper and many others.

In the past years, there have been significant advances in the characterization and modification of starches, boosting their versatility and their applications beyond food, attracting the interest of different industrial sectors. However, in spite of the considerable advances in the field, starch's structure is still not completely understood. In fact, despite being formed mainly by glucose units, both molecular and granular structures of starches are highly complex, requiring more studies. Furthermore, most of the industrial modification processes of starches involves chemical agents, going against the increasing consumer's concerns regarding food safety and environmental impacts.

In other words, starches complex structure, added to the fact that their behaviour under different processing conditions is sometimes unpredictable, indicates that there are still some gaps regarding their understanding and characterization. In addition, the usually performed modification processes no longer meet the growing demand for less environmentally harmful technologies. Consequently, starches and starch modification processes are still widely investigated worldwide.

All those issues inspired us to deeply explore starches from several sources, including their behaviour under different processing conditions and after subjected to more environmentally friendly modification technologies. In fact, this work started with my Master's research [1], where the potato starch modification using the ozone technology was evaluated. By using ozone at different treatment times, it was possible to assess the effect of this gas in the potato starch structure (both molecular and granular) and in some of its functional properties [2].

After finishing my Master's degree, other questions were raised, such as: how the starch structural modification affects its properties? What new applications can be proposed for starches with those obtained properties? How all of this impacts the starches' stability and behaviour under different processing and storage conditions? On the other hand, how the analyses conditions affect the starch properties? What is

the best way to evaluate those properties? Can standard analyses be changed to better describe the starch properties? If so, how can we compare the results? If interesting results were achieved using ozone technology, how about using other emerging technologies, alone or in combination?

To answer these questions, we decided to continue our investigations about starches, in order to better understand the impact of their structure, as well as the processing and modifying conditions, on the observed properties. In this context, my Ph.D. Thesis was developed, intending to evaluate the structure and the properties of starches from different sources, subjected or not to modification processes, in order to enhance their performance for further applications. For that end, the obtained starches were assessed using both standard and non-standard conditions of analyses.

The present Thesis resulted in six articles (Appendix from A to F), which are organized in two main groups (non-modified and modified starches), as illustrated in Figure 1, being developed with the partnership of other institutions and researchers from Brazil, Peru and USA.

In the first group, the performance of non-modified starches was evaluated, meeting the consumers' search for more "natural" products. For that end, in the first work of this part, starches extracted from two varieties of arracacha, a starch-rich root from Peru, were characterized and described for the first time (Appendix A). On the other hand, in the second work, the effect of the processing conditions (in special the starch concentration) on the functional properties of five well-known native starch sources (cassava, potato and three types of maize starch: normal, waxy and high amylose) was explored (Appendix B).

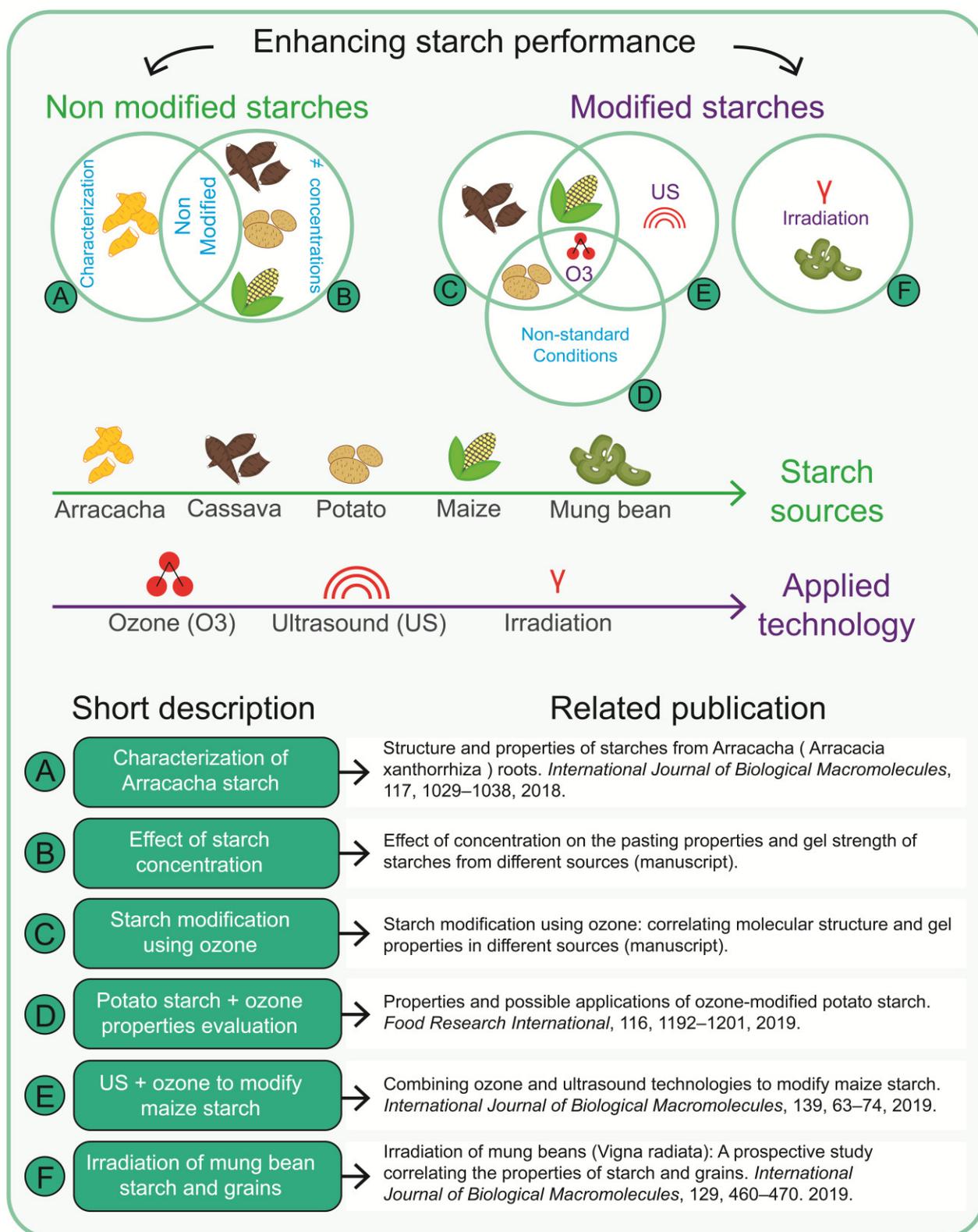


Figure 1. Diagram of the Thesis organization, illustrating the two main groups of studies (enhancing starch performance using both non-modified and modified starches) and describing the six resulted articles (Appendix from A to F).

On the other hand, in the second group, starch modification using emerging technologies was evaluated. Firstly, the effect of the ozone technology on different starch sources (cassava, maize and potato) was evaluated, being the molecular features and pasting and gel properties of those starches analysed using different approaches (Appendix C). Then, the ozonated potato starch was selected to be further evaluated using different conditions, in order to simulate different industrial processes (Appendix D). Furthermore, as the combination of technologies is rarely assessed in the literature, the combination of ozone with another emerging technology – the ultrasound – was performed on maize starch (Appendix E). Finally, a third emerging technology was evaluated (gamma irradiation), and its impact on both mung bean starch and grains was analysed (Appendix F).

2. LITERATURE REVIEW

2.1. Starch – general characteristics

Starches, besides being composed mainly by glucose units (in the α -D-glucopyranosyl form), are a very complex material, since these units are linked to form the two main polysaccharides that compose the starch granules: amylose and amylopectin. These two macromolecules present different structures and their composition and ratio greatly affect the starch properties. Besides, they are organized in rigid and insoluble structures called granules, which presents a double-helical, semi-crystalline and birefringent structure. Different botanical sources present starches with different molecular and granular characteristics; consequently, distinct properties are available depending on the starch source [3–6].

The amylose molecules are composed mainly by (1→4)-linked α -D-glucopyranosyl units, which gives them a linear arrangement, with few (1→6)-linked branches. It is believed that the amylose molecules are randomly mixed with other materials in the amorphous region of the starch granule. On the other hand, the amylopectin molecules are highly branched, being composed by short chains of linear units. The branched aspect of the amylopectin molecules gives them an arrangement of double helices, composing the amylopectin clusters, being the main component of the crystalline regions of the starch granule [6,7]. Figure 2 brings a schematic representation of the amylose and amylopectin molecules.

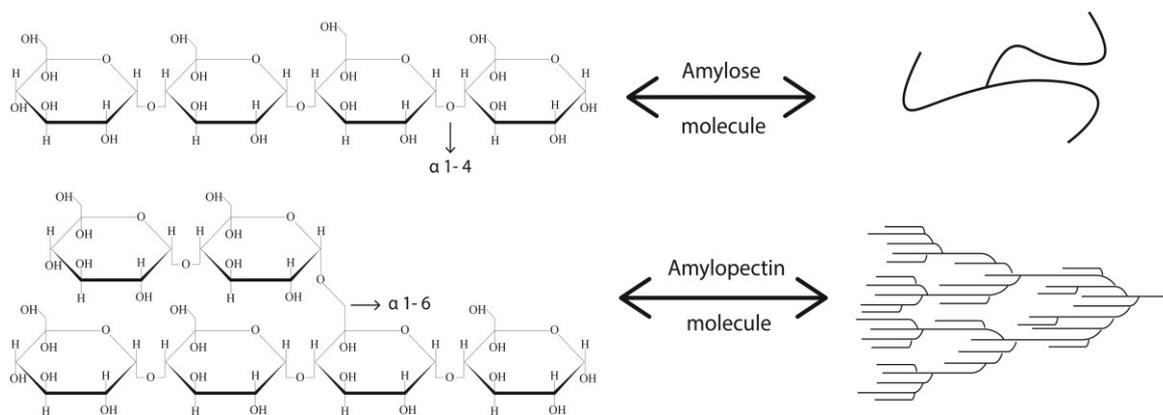


Figure 2. Representation of the amylose and the amylopectin molecules.

The complexity of the starch granules, however, goes beyond the amylose and amylopectin molecules. The starch granules are normally formed by a hilum at the centre, which is surrounded by amylose and amylopectin molecules arranged radially in a “blocklet structure”, presenting well defined amorphous and crystalline regions. A comprehensive illustration of the structure of a starch granule can be found in Figure 3, which was inspired in literature information presented in several works [2,3,7–12].

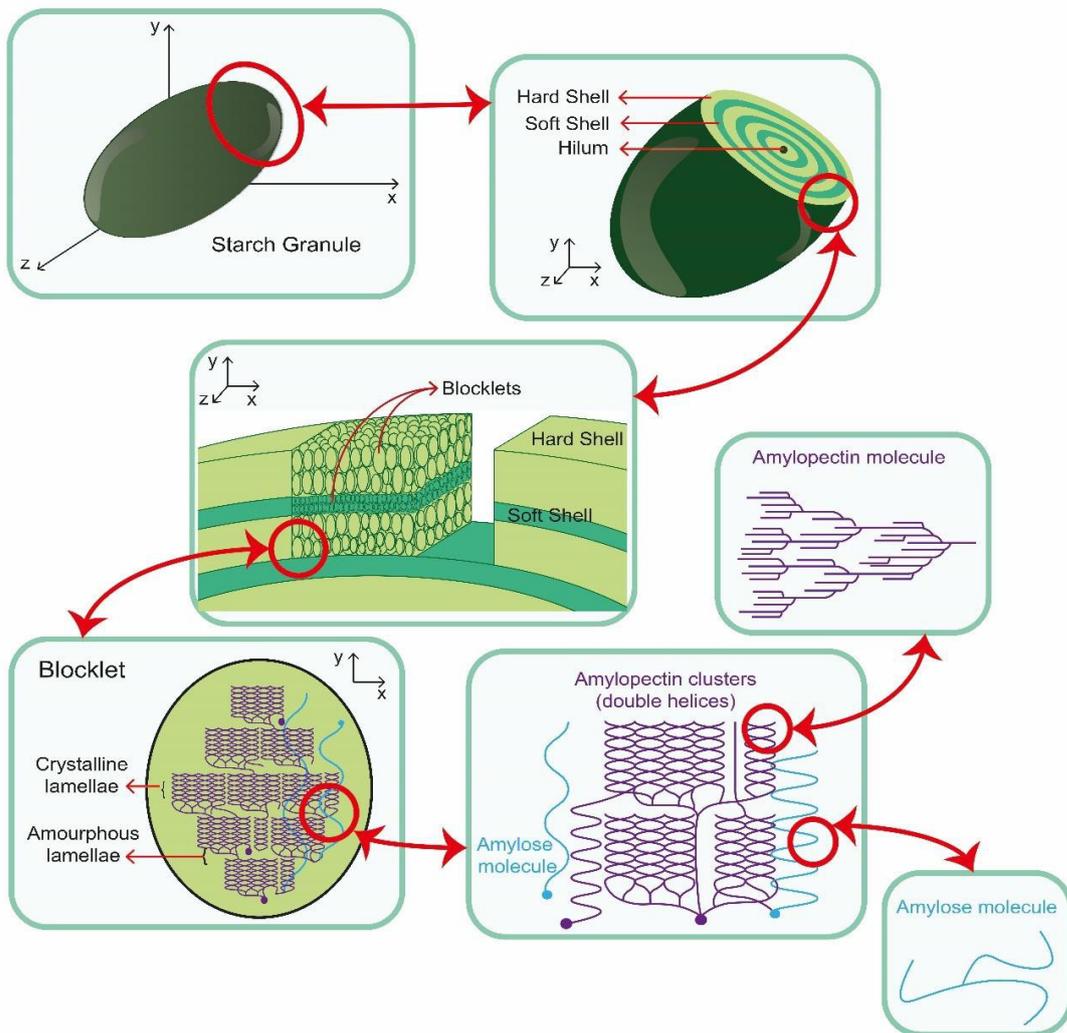


Figure 3. Representative illustration of the parts that compose a starch granule.

Since the starch structure is complex, its behaviour under different process conditions is difficult to predict and to evaluate. Regarding its properties, the most important considering the industrial point of view are the starch pasting and thermal properties. Both are closely related to the starch gelatinization process, which is quite complex and difficult to measure even on a laboratory scale [3,13], further discussed.

2.2. Starch gelatinization

Starch gelatinization occurs following some steps.

At temperatures higher than the gelatinization temperature (known as onset temperature), the intermolecular bonds that maintain the crystalline structure of the granules undergo a natural weakening, allowing the water molecules in their vicinity to penetrate their rigid structure and to bond with the molecular hydroxyl groups. At this stage, the starch granule swells and loses its birefringence. With the gradual temperature increasing, the granular swelling also increases, and the granules become more and more susceptible to shear disintegration [14]. An illustrative representation of the steps of starch gelatinization is shown in Figure 4, which was initially published in our article attached to the Appendix F [15], being based on the theories presented in the works of Atwell et al. [16], Liu [3] and Xie et al. [14].

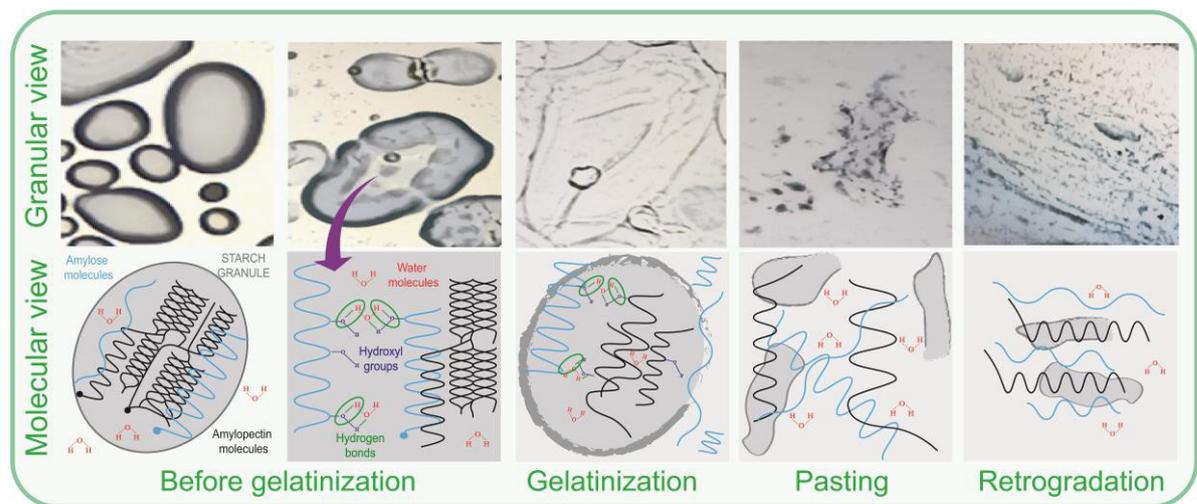


Figure 4. Graphic representation of the starch granules (top line) and molecules (bottom line) in different steps of a gelatinization process. The top line represents a granular view using actual images of a potato starch granule. The bottom line brings a schematic representation of a molecular view.

It is important to mention that starch gelatinization is one of the most explored phenomena in starch science, and it has been explored in both classical [17–21] and current literature [22–24].

However, despite the massive amount of works on this subject, and despite the different techniques employed aiming to better characterize the gelatinization behaviour of different starch sources, this phenomenon can still be unpredictable in

different conditions. An evidence of this unpredictability is the raising of recent articles whose purpose is to model the gelatinization stages of different starches [25,26], which proves that it is a still unsolved problem.

There are different methods to study and to evaluate the starch gelatinization, but all currently known methodologies have advantages and disadvantages and are, to a greater or lesser extent, influenced by some factors. In fact, aspects as starch concentration, granule type, homogeneity of the granular population, temperature and heating rate, shear forces, among others, make the gelatinization process of starches difficult to predict and control [3,13,25].

Meantime, the importance to understand the gelatinization process and how it affects the starch properties goes beyond the scientific approach. For instance, mostly starchy systems undergo mechanical and thermal processes before being used, since at native conditions they are insoluble in water at room temperature and less digested by the human body [27]. Besides, to achieve the required characteristics for different applications, the thermal processing of starch samples is performed at different conditions, which vary widely among industries and among products. Consequently, most of the industrial applications involve gelatinized starches, or their gelatinization.

Therefore, the functional properties of different starchy food products, as their texture and digestibility, are closely related to the gelatinization and retrogradation processes. In this manner, the standard conditions of analyses may not be representative for all industrial applications, thus evaluations at different conditions are important to better characterize starches. Nevertheless, despite the complexity to estimate and to measure the starch properties, especially under heat and shear, this material attracts much academic and industrial interest, and its properties are still evaluated by different researchers worldwide.

2.3. Botanical sources of starches and their industrial applications

In food and non-food industries, like pharmaceuticals, textiles, paper, mining and adhesives, starches have wide application, since they are abundant, cheap, versatile and they can be obtained from renewable and biodegradable sources [28]. However, the starch of each botanical source has specific properties, since the amylose and the amylopectin molecular size distribution and ratio, as well as their spatial conformation, vary widely within the starch granules. Consequently, the

selection of a specific starch source – or of a particular modification technique or processing condition, as will be further described in this Thesis – is necessary in order to provide the desirable properties for the different industrial applications.

Considering starch-rich botanical sources (being the most important the tubers, roots, cereal grains and legume seeds), the highly commercially exploited ones, such as potato, cassava, maize and rice, among others, have their respective characteristics and properties well described in several studies [3,29]. On the other hand, a diversification of works investigating the properties of starches from different starch-rich vegetables is raising and gaining attention, as Andean and Amazonian tubers and roots [30–32], African rice [33,34], barley [35], common beans [36], among many others. In all that cases, there is an inherent advantage: the high starch content, increasing the yield of the extraction processes.

Nevertheless, the evaluation of unconventional sources of starch and their applications has been observed in the literature, such as pumpkins [37], cashew nut shells [38], black pepper [39], pineapple wastes [40], mango kernels [41], banana [42], pinhão [43], kiwifruit [44], babassu [45], among others. In many of these cases, the sources present a difficult starch extraction and a low yield. Consequently, the search for unusual properties in natural sources surpasses the possible low industrial feasibility.

However, despite the many possibilities of new and underexplored starch sources, the properties of the native starches and/or of starches from popular commercial sources may not correspond the industrial needs. In this Thesis we proposed two possible alternatives to work around this issue, aiming to achieve characteristics that better suits the industrial applications.

The first alternative is the investigation of non-standard analyses conditions using popular starch sources. Although the starch characterization is important, since the difference among starches' composition and fine structure implies in significant changes in their properties, understanding how the processing conditions influences the starch properties is of similar relevance. Most of the studies regarding the characterization of starches do not consider different processes conditions, being analysed only under standard settings. As a result, valuable information about the starch properties are lost. Therefore, in this Thesis, the influence of non-standard conditions of analyses on starch properties was evaluated.

The second alternative to obtain starches with better characteristics is by modifying them, which is better described in the next section.

2.4. Starch modification

Starch modification is widely employed in the industry, since it is a viable form to provide new characteristics to the native starches, improving the products' functional properties and bringing competitive advantages to them [46]. According to Kaur et al. [47], there are four kinds of starch modification: chemical, physical, enzymatic and genetic.

Enzymatic modification is based on the use of different enzymes, being the most common the hydrolysing ones, while the genetic modification is based on the production of novel starches through a transgene technology. Amylose-free (waxy) starch, high-amylose starch and starches containing higher or lower phosphate content are some examples that can be obtained from transgenic sources [47].

Physical modification methods, such as ultrasound, irradiation, pulsed electric fields (PEF), microwave and extrusion, among many others, are being used to produce modified starches with specific functional properties, with the positive aspect of not involving any chemical presence. In contrast, the chemical modification methods are recently rising issues regarding consumers and the environment, since they usually present a consequent waste generation and high costs, which leads to a search for alternative modification processes [47,48].

In this Thesis, one alternative chemical technology (ozone) and two physical methods (ultrasound and irradiation) for starch modification were evaluated.

2.5. Ozone technology and its use for starch modification

The ozone (O_3) gas is obtained through the oxygen molecules (O_2), being synthetically generated by the exposure of air, or another oxygen-rich gas, to a high-energy source, which convert the oxygen molecules to ozone molecules. Ozone is partially soluble in water, has a pungent and very characteristic odour, is relatively unstable at normal temperatures and pressures and is a very powerful oxidising agent (electrochemical potential = +2.075 V) [49,50].

Ozone was recognized as a Generally Recognized as Safe (GRAS) product by the Food and Drug Administration (FDA - USA) in 1997 [51]. Since its permission

to be used as a direct contact food sanitizing in 2001, the ozone has been used in several applications by the food industry [50,51]. If compared to other chemical and oxidising agents, this gas has the advantage of being a green and environmentally friendly technology, since it does not leave any residues in food or in environment, being quickly decomposed to oxygen [52].

All those characteristics make the ozone technology a good alternative for the starch modification using chemical agents, such as sodium hypochlorite and hydrogen peroxide. Those agents' evident drawbacks, as the low process yield [53] and, mostly important, the large generation of wastewater containing high concentrations of salts or toxic wastes [48] should be enough to decrease their use. However, in the starch industry, the ozone technology is still not popular.

In fact, despite the many advantages related to the ozone technology, starch modification using this gas still presents some gaps that need to be overcome. Therefore, more studies about the ozone effect on the starch samples are necessary and are gaining attention. In this context, ozonation is being used to modify a wide range of starch sources, such as corn, sago, tapioca, cassava, rice, wheat and potato, among others [2,48,61,52,54–60].

It is important to mention that the main points regarding the ozone effect on the starch structure are fairly elucidated. Two main reactions take place simultaneously during ozonation process of starch. The first one is the oxidation of the hydroxyl groups of the starch molecules, generating carbonyl and carboxyl groups (Figure 5). The second is the depolymerization of the starch molecules by the cleavage of the α -(1→4)-glycosidic linkages [46,62]. Consequently, molecules with different size and distribution, charges and chemical affinity are obtained.

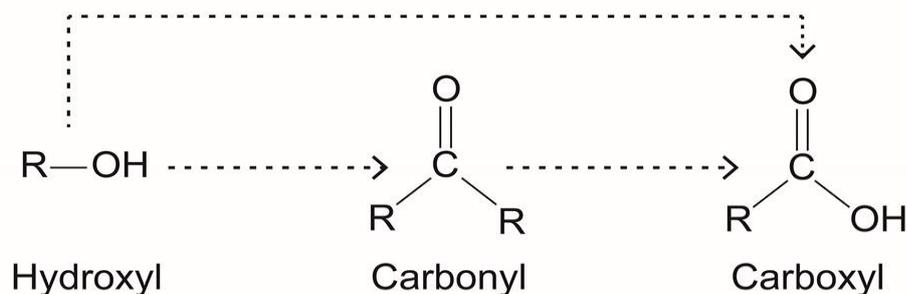


Figure 5. Hydroxyl groups oxidation into Carbonyl and/or Carboxyl groups.

However, despite those changes (formation of electronegative groups and molecular hydrolysis) be similar for all starch samples, the way of how they affect the starch properties is still not completely understood. In fact, it is important to highlight that the botanical source and, consequently, the structure of the starch influences the ozone action and the obtained properties, being those aspects of high relevance. Furthermore, as far as we known, the mechanisms of action of the ozone combined with other technologies, as the ultrasound, were not yet explored. Therefore, in this Thesis, the effect of the ozone processing on different starch sources was evaluated, being correlated with their structure, the observed properties and with possible applications. In addition, the combination of the ozone technology with the ultrasound – a physical modification process – was analysed.

2.6. Ultrasound technology and its use for starch modification

The sound can be described as mechanical waves generated by a vibration of a given material, resulting in a variation of pressure in a gaseous, liquid or solid system. In turn, the ultrasound is based on the application of this acoustic energy at high frequencies (higher than audible frequency, usually >18 kHz), being a physical process [63,64].

The ultrasound can be classified as low intensity ultrasound (frequencies higher than 100 kHz at intensities below 1 W/cm²) and high intensity ultrasound (intensities higher than 1 W/cm² at frequencies between 18 and 100 kHz). Low-intensity ultrasound, which can be used for non-invasive monitoring of food processes, is generally non-destructive, causing no physical or chemical alterations in the properties of the materials. On the other hand, the high intensity ultrasound can change the material properties through different mechanisms, being the most mentioned ones the “sponge effect” and the acoustic cavitation [63,65,66].

Being the sound a variation of pressure, when a material is subjected to an ultrasonic process, mechanical waves traverses it, resulting in compression and expansion cycles. In the expansion cycles (i.e. a low-pressure condition), there is a formation of microbubbles in the system through vaporization. In turn, in the compression cycles (i.e. a high-pressure condition) a condensation of the formed bubbles occurs. Along this alternation of compression-expansion cycles (known as

“sponge effect”), those bubbles can collapse in a process involving high temperature and shear forces (known as acoustic cavitation) [63,65,67–69].

The ultrasound has been successfully applied to optimize several industrial processes, such as homogenization [70], stabilization [71], mass transfer [72–74], and preservation [75], among others.

The use of ultrasound for the modification of structural and technological properties of starches is also being reported in the literature, being applied in different starch sources, such as rice [76], oat [77], cassava [78], maize [79,80] and potato [81,82], among others.

The main described structural changes caused by the use of ultrasound in starches are the modification and disintegration of granules [81–83], and molecular size reduction [83,84]. Such changes are reported to be caused by both acoustic cavitation and by the highly unstable free radicals formed in the implosion of bubbles in the systems in the presence of water [84,85].

However, structural modifications of biopolymers by ultrasound treatment are reported to be highly dependent of the equipment’s characteristics (i.e. frequency, intensity) and the system’s properties (i.e. viscosity, temperature, pressure) [86,87].

In fact, some of the results presented in the literature are contradictory. These differences in the observed results emphasize the lack of standardization during these treatments and prevents more precise statements and conclusions about the ultrasound efficacy and its effects on the starch molecular structure. Moreover, some works in the literature do not follow good practices in ultrasound processing, which can compromise the obtained results [88].

Finally, the combination of ultrasound and ozone to modify starches have been little explored. Only two published works explored this subject, being published while we were developing this idea independently [89,90]. However, both papers did not provide information on structural changes neither potential correlations between the modified starch structure and its properties. Consequently, the mechanisms of the combined action of ultrasound and ozone on starch remained unexplored. Therefore, in this Thesis, the structure and the properties of modified maize starch using combined ultrasound and ozone treatments were evaluated.

2.7. Irradiation technology and its use for starch modification

The term “irradiation” is used to refer to processes where the material is exposed to an ionizing energy. In the industry, materials can be exposed to different types of irradiation, as for example through microwave-ovens or X-ray equipment. Another way is by using gamma photons emitted by radioisotopes (usually ^{60}Co). The main difference between those irradiation sources is related to their penetration ability. Usually, the absorbed radiation doses are quantified in kilo gray (kGy) units (1 kGy = 1 J/kg) [91]. Therefore, irradiation is a high-energy technology, being also an effective and underexplored emerging technique with different applications in food processing.

It is important to stress that the irradiation methods, although involving ionizing radiation, do not generate radioactive foods. It is a physical treatment which involves a direct exposure of the product to electromagnetic rays, aiming to improve its properties and safety [92–94]. In fact, after decades of study, the food irradiation was proved to be safe and it is a recognized food technology around the world [95]. In fact, there are several advantages related to this technology: it is fast, requires minimal sample preparation, is a “cold” process, versatile and is highly effective and penetrating [95–98]. The principle of action of this method is based on the generation of free radicals, which can cause modifications on the exposed materials [98]. However, although there is a vast literature regarding the use of irradiation for microbial inactivation, the effect of this technology on technological properties of food still needs further studies.

Furthermore, despite several works have reported changes on the structure and properties of different starch sources, such as rice [99,100], chickpea [97], potato [101,102] and maize [102], the results do not always follow the same behaviour, probably due to the different starch sources and/or irradiation dose. Despite the contradicting results, the most mentioned effects related to the irradiation process are the hydrolysis of glycosidic bonds and the consequent depolymerization of the starch molecules, and the increase of the carboxyl content and decrease of the apparent viscosity during the starch pasting [99–101].

In this Thesis, we evaluated the effect of gamma-irradiation on the characteristics of mung bean (*Vigna radiata*) starches (structure and properties), as well as correlated the starch properties with the grains’ properties (hydration, germination and texture), being both approaches described for the first time here.

3. GENERAL OBJECTIVES

The main objective of this Thesis was to enhance the performance of different starch sources aiming to improve their industrial applications. To that end, the structure and the properties of starches from different sources, subjected or not to modification processes, were evaluated using both standard and non-standard conditions of analyses.

Based on this primary objective, some specific objectives were designed:

- To characterize a new starch-rich source (arracacha root), aiming to boost its commercial exploitation and to obtain desirable functional characteristics from a natural starch source;
- To explore the properties of commercial starch sources using both standard and non-standard conditions of analyses, in order to expand their versatility without resorting to modification methods;
- To modify different starch samples using emerging technologies (ozone, ultrasound and irradiation), and to correlate the processing conditions, the starch structure and the obtained properties, proposing new applications for the obtained samples.

4. THESIS DEVELOPMENT AND GENERAL RESULTS

As explained in the section “1. Introduction and Thesis organization” and illustrated in Figure 1, this Thesis is divided in two parts: enhancing starch performance using (1) non-modified and (2) modified starches.

Each part was developed aiming to better explore a specific approach of starches’ characterization, being formulated based on questions raised after evaluating previous works (ours and from the literature). The main pros and cons related to each part is summarized in Figure 6. Then, a summary of the main results and discussions of the articles that compose this Thesis is further presented.

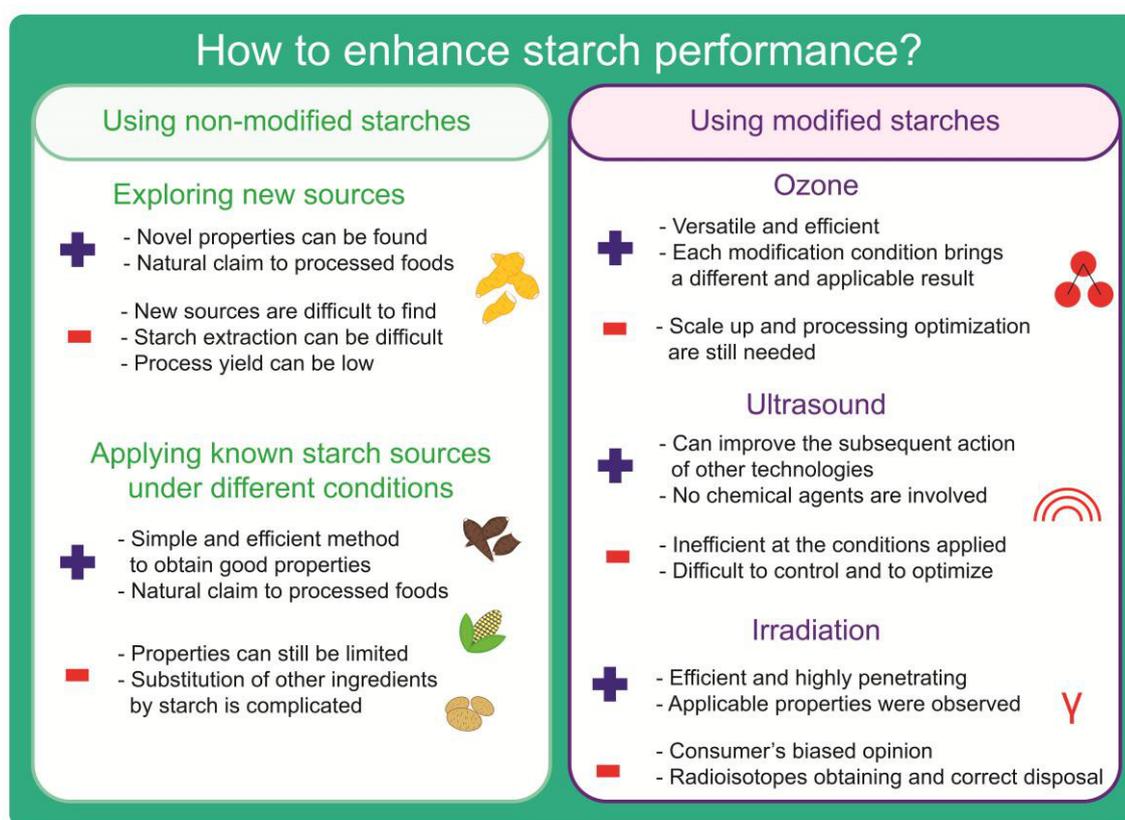


Figure 6. Summary of the main pros and cons related to each part of the Thesis.

4.1. Expanding the possibilities of application of non-modified starches

This part intended to boost the applications of starches in their native form (non-modified), considering the claim for more natural products, using two approaches: evaluating starches from a non-explored (new) starch source (Appendix A), and exploring well-known starch sources, but using non-standard conditions of analyses in

order to better simulate industrial applications (Appendix B). The main results of each article are described below.

4.1.1. Characterization of starches from a novel source: the case of the Andean arracacha root

In this article, we extracted and characterized starches from two varieties (*amarilla* and *morada*) of arracacha (*Arracacia xanthorrhiza* Bancroft) root, an underutilized and starch-rich source from the Andean region of Peru (Appendix A). This work was made jointly with the “*Universidad Nacional Agraria La Molina*”, of Lima (Peru).

The two varieties of this root were selected to be characterized for several reasons. The first and most evident one was the fact that the starch from those roots were not evaluated before. The second and most important was the arracacha’s high starch content (more than 50% of its dry matter), with relatively simple extraction process. Finally, boosting the arracacha’s commercial value would be meaningful to its production region, by giving a higher importance to this underutilized and highly perishable starch-rich root [103–105].

To evaluate the arracacha starch, several analyses were performed, in order to characterize its structure (granular and molecular) and functional properties.

Considering the granules’ characteristics, the arracacha starch presented small-sized granules (~20 μm) with a B-type crystalline structure, organized in a compact (“lego-like”) formation. The starch was also rich in amylopectin molecules with long chain lengths and high ability to complex iodine.

However, it was the functional characteristics of the arracacha starch that stood out, for being rare in a native starch, and for presenting great possibilities of industrial applications. Among them, we highlight its low gelatinization temperature (~53 to 55 °C) and enthalpy of gelatinization (~1.9 to 2.6 J/g). In fact, if compared to other starch samples, such as maize (64.1 °C; 12.3 J/g) and cassava (64.3 °C; 14.7 J/g) starches [19], those values were even more impressive. These gelatinization characteristics reflected in the water absorption capacity of the arracacha starch samples, which was remarkable even at moderate temperatures (60 °C), being able to absorb and retain ~12 g water/ g starch. As a basis of comparison, potato starch evaluated under the same conditions (60 °C) absorbed less than 4 g water/ g starch.

Moreover, the arracacha starches presented pastes with high peak apparent viscosity, which also presented high paste clarity (> 90% of transmittance) and a very low retrogradation tendency. In fact, the gels presented no syneresis even after 15 days of refrigerated storage, being this a remarkable characteristic. All those results, summarized in Figure 7, illustrates the fact that the arracacha starch presents interesting industrial properties, especially regarding the food industry, since using starches in their native form gives a “natural” claim to processed foods. Possible applications for each property were also discussed.

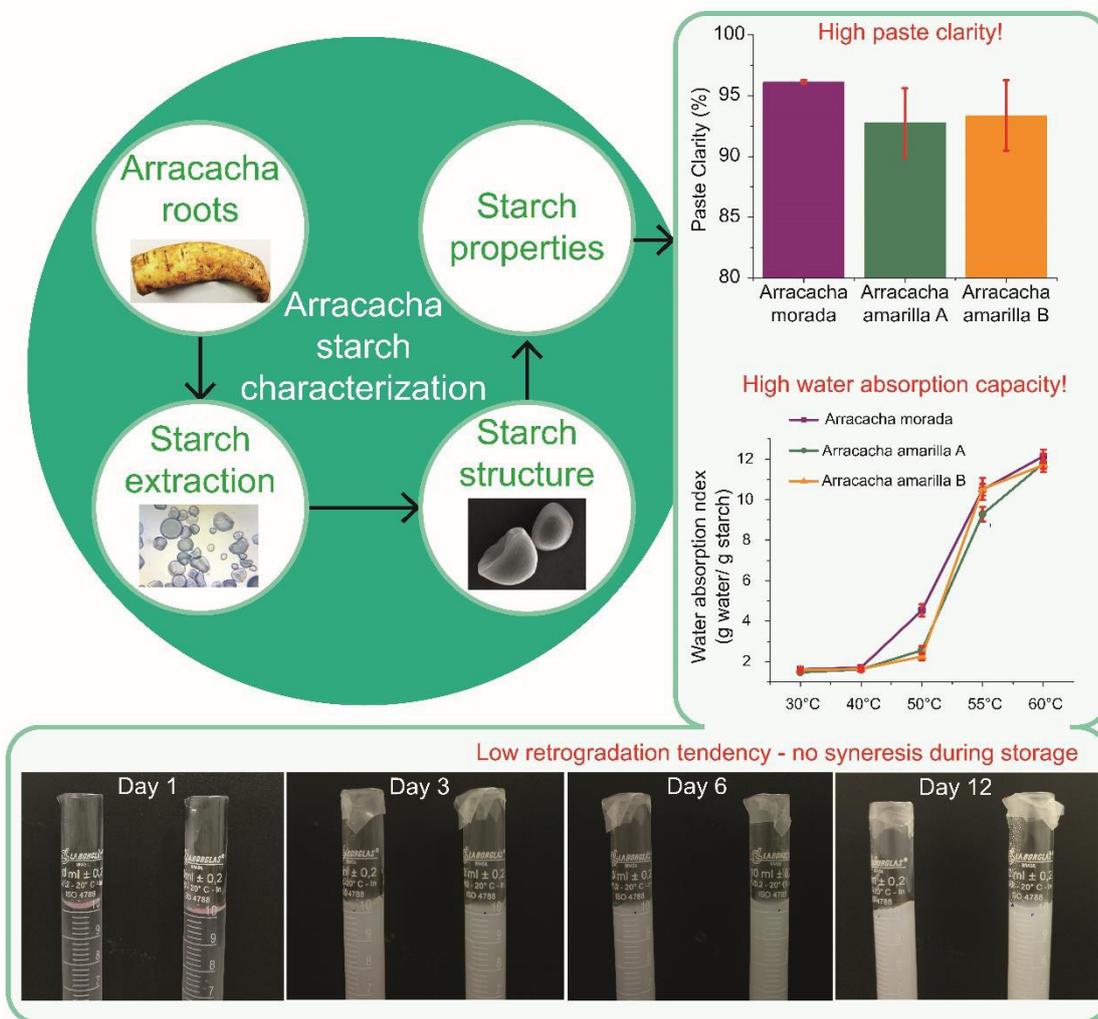


Figure 7. Schematic representation of the main steps of the arracacha starch obtaining and some of its remarkable characteristics.

Summarizing, starch samples from Andean arracacha roots were extracted and characterized for the first time in this study. In addition, the structure features of the samples were correlated with the observed properties, which in turn were

remarkable for a native starch sample. Therefore, the obtained results were published in the article entitled "[Structure and properties of starches from Arracacha \(*Arracacia xanthorrhiza*\) roots](#)" by the International Journal of Biological Macromolecules, presented in Appendix A.

Nevertheless, as new and underexplored starch-rich sources are difficult to find nowadays, and as we consider that exploring sources that present a difficult starch extraction can be counterproductive, the next study was focused on evaluating the effect of different processing conditions on well-known native starch sources.

4.1.2. Characterization of different starch sources using non-standard analyses conditions

In the second article of this first part of the Thesis, the pasting and gel characteristics of native starch sources were evaluated using non-standard conditions of analyses (Appendix B). The main objective of this work was to report how the processing conditions interfere with the obtained properties of native (non-modified) starches, as well as illustrate how their behaviour under those conditions can be sometimes unpredictable. To that end, well-known starch sources (cassava, potato and three types of maize starch: normal, waxy and high amylose) were selected in order to allow a fair correlation between their structure (vastly reported in the literature) and the observed properties.

We are aware that, as those sources were considerably studied, their pasting properties and gel characteristics have already been previously reported by different authors. However, when we searched in the literature for works reporting the gelatinization behaviour of the mentioned sources at low concentrations (lower than 10.7%, which is the standard for RVA analysis but is too high for most industrial applications), all under the same conditions, we could not find none. In fact, until this moment, the studies found in the literature were carried out using only one starch source, or different sources but under standard protocols, which difficult to make comparisons, interpretations and lead to incorrect conclusions. Therefore, in this article we explored four different starch/water concentrations and two different gelatinization temperatures, as illustrated in Figure 8. The starch proportions were based on the RVA protocol (total of 28 g of sample), being established as: 3.6% (1 g starch + 27 g water),

7.1% (2 g + 26 g), 10.7% (3 g + 25 g, being this concentration used in the standard protocols of the RVA equipment) and 14.3% (4 g + 24 g).

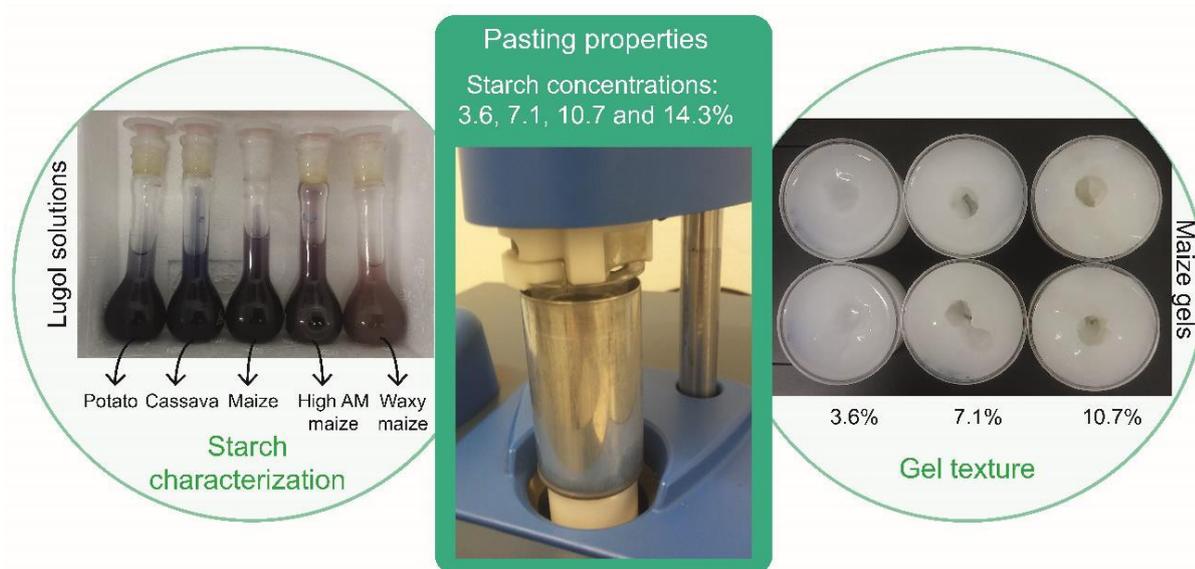


Figure 8. Schematic representation using actual images of the main analyses performed in the article (Appendix B).

It is important to emphasize that, despite the approach of this article being simple, the obtained results are unexplored and relevant, as we are providing new data for those starch sources characterization and application. In fact, we demonstrated with our results that when the starch concentration varies, its behaviour is completely different and sometimes unpredictable, considering both pasting and gel characteristics. For example, potato starch presented a “non-standard” shape in its pasting curve at concentrations close to those typically used in food industry (3.6%), without the characteristic “Setback” and “Breakdown” regions, when compared to the standard protocol of evaluation (10.7% of concentration). As another example, the waxy maize is usually mentioned as presenting a higher Peak apparent viscosity if compared to the normal maize starch. However, this statement can only be considered true at the lower evaluated concentrations, since at highest concentration the normal maize presented a higher Peak apparent viscosity value if compared to the waxy sample. All those differences among the pasting properties results at different conditions are shown in Figure 9.

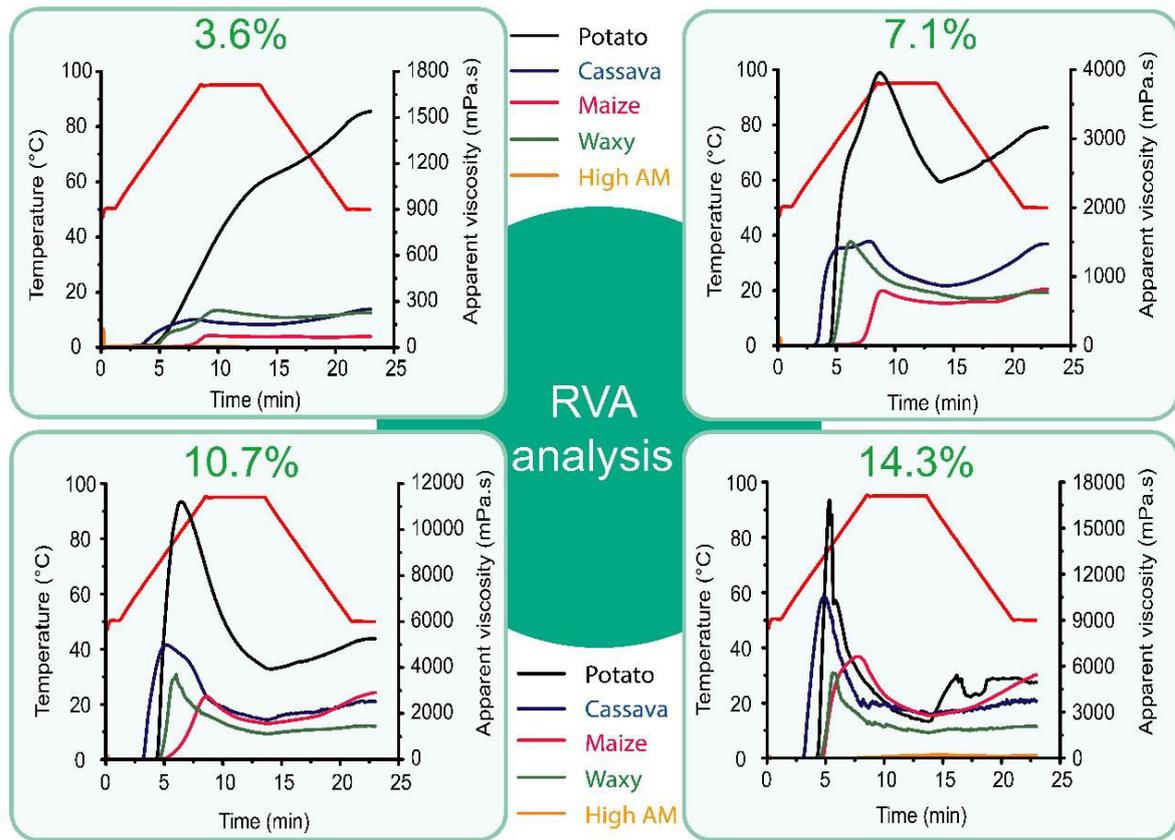


Figure 9. Pasting properties of native potato, cassava, maize, waxy maize and high amylose (AM = amylose) maize starches, using four different starch concentrations (3.6%, 7.1%, 10.7% and 14.3%).

In summary, we highlight that decisions of industrial application based on the results of standard protocols may not suit the real necessities, leading to misinterpretations. Furthermore, the conditions in which a starch sample will be applied in a product must be considered as important as the starch source itself. Therefore, as the results obtained in this study are relevant and still unpublished, they are described in the article “Effect of concentration on the pasting properties and gel strength of starches from different sources”, to be soon submitted to the Carbohydrate Research journal, and showed in Appendix B.

4.1.3. Conclusions and future works

Considering the main discussions of the first part of the Thesis, we can conclude that exploring new starch-rich sources can be a good alternative to find novel functional properties for industrial applications, without resorting to modification processes, as the case of the arracacha starch (which presented very promising functional

properties). As future works in this line, therefore, we can suggest further characterization of new botanical sources or even of starches from agroindustrial wastes, as it was already done with pineapple stem [40] or mango kernels [41], which would be a good alternative to take advantage of these materials. However, this approach can be challenging, since unconventional starch sources are rarely found. Besides, exploring unusual sources can be counterproductive if the starch is difficult to extract or if the yield of the process is low.

On the other hand, by using widely known starch sources under different processing conditions (as varied concentrations and temperatures), it is possible to obtain diverse and more applicable properties. However, those properties are still limited. Furthermore, other issues can restrict this approach, as food legislations (which can limit the use of starches as ingredients or additives in some products), or a possible recipe imbalance by replacing other ingredients by starches. In any case, the exact role that each parameter plays (related to the starch structure and to the processing conditions), and how much they influence the final characteristics of starchy products, remain unclear. Therefore, future works using different analysis conditions are still important in order to better correlate the relationship between the starch structure and properties with the processing conditions. Another possibility would be to model starch samples' behaviour as accurately as possible under the most varied conditions, thus enabling a more precise decision of which conditions to apply for each case, without the need for an empirical assessment.

Nevertheless, all these mentioned issues illustrate that starch modification is still necessary in some cases. However, alternative methods are needed in order to replace the conventional and more environmentally harmful ones. Therefore, the second part of this Thesis was focused on exploring and expanding the knowledge about starch modification using different emerging technologies.

4.2. Starch modification using emerging technologies

In the second part of this Thesis, starch modification using three different emerging technologies, namely ozone, ultrasound and irradiation, was evaluated. A greater focus was given to the ozone technology, which was the basis of three different articles. The first one explored the ozone technology to modify cassava, maize and potato starches (Appendix C). In the second one, the effect of the ozone processing

on the properties of the potato starch was deeply evaluated using non-standard conditions of analysis (Appendix D). And the third one evaluated the combination of the ozone and ultrasound technologies to modify maize starch (Appendix E). Finally, the irradiation technology was used to modify mung bean starch in the last article related to the second part of this Thesis (Appendix F). The main results related to each article are described below.

4.2.1. Expanding the knowledge about starch modification using the ozone technology: a prospective study using cassava, maize and potato starches

This work (Appendix C) intended to better explore the effectiveness and the versatility of the ozone technology, by evaluating the structure and the properties of different starch sources (potato, cassava and maize) under the same processing conditions, thus allowing comparisons among them. The ozonation system used for this purpose is illustrated in Figure 10.

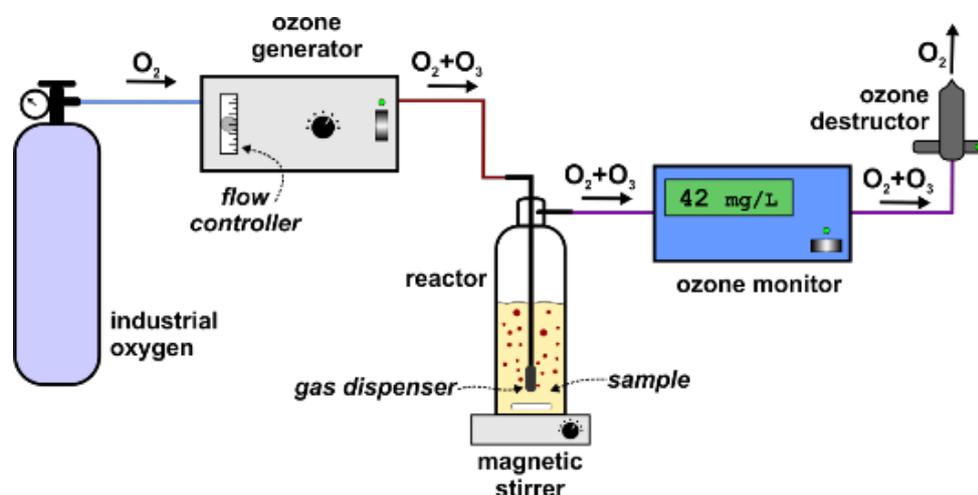


Figure 10. General aspect of the ozonation system used for starch modification

The ozonation conditions were selected based in the results of our first work involving starch modification using ozone [2], where potato starch was modified using different ozonation times: 15, 30, 45 and 60 min. However, in our previous work the 45 and 60-min samples resulted in very weak gels. Therefore, for the present work, we have chosen to treat the starch samples only up to 30 min of ozonation, in order to better explore the gels' characteristics. To that end, the functional properties of the gels

were evaluated using distinct analyses (pasting properties, and viscoelastic and texture properties of the gels before and after their refrigeration and storage) and different starch concentrations (5 and 10.7%).

Furthermore, in our last work, some analyses were performed in order to better understand the effect of the ozone on the starch structure, and a possible mechanism of action of the ozone for starch modification was proposed. However, most of the previous analyses were not conclusive by themselves, and a combination of them was required to reach a conclusion about the observed structural changes. Therefore, in this present work, we chose to perform a more refined analysis that would be more conclusive about the observed structural modifications. To that end, we worked in partnership with the “Purdue University” (USA) – which has one of the world's most renowned and equipped centres for carbohydrate analysis, the Whistler Center for Carbohydrate Research. During my 6-month period there, I was able to learn and perform more specific chromatographic analysis for starch samples, which allowed a better understanding of the molecular changes undergone by the ozonated samples.

Therefore, the starch molecular size distribution profile was evaluated using two chromatographic techniques: a high-performance size-exclusion chromatography coupled with multiangle laser light scattering and differential refractive index detectors (HPSEC-MALLS-RI), and the gel permeation chromatography (GPC) based on iodine affinity (blue value – BV). While I have used the GPC before [2,106], once it is available in our research group, it was on Purdue University that I could learn and use the HPSEC-MALLS-RI system. Interestingly, the results obtained by both techniques led us to the same conclusions: an overall reduction of the sizes of both amylose and amylopectin molecules.

In addition, the macromolecular results obtained through the HPSEC-MALLS-RI system confirmed the molecular depolymerization caused by the ozone treatment (reduction in the values of molar mass and gyration radius) and indicated a less homogeneous molecular weight distribution of the samples (higher molar-mass dispersity).

Furthermore, the molecular analyses illustrated an outlying behaviour of the maize starch (named as “corn” in the manuscript, Appendix C), which presented an apparent “resistance” to the ozone action, taking longer time to be affected if compared to potato and cassava samples. After evaluating different characteristics of the starch

samples, such as granule size and amylose/amylopectin content, this outlying behaviour was related to the higher complexed lipids content of the maize starch [107], especially the ones placed on the granules' surface, impairing in some extent the ozone action.

Regarding the starch properties, they were also affected by different factors, and each source presented a particular behaviour. In fact, all the observed properties (summarized in Figure 11) illustrated the complex balance between the ozonation conditions, the starch source (and consequently its structure) and the analysis conditions (especially regarding the starch concentration).

For instance, the RVA results for the three starch sources were very different from each other, even when the same ozonation and analysis conditions were applied. Furthermore, those results proved that evaluating only one starch concentration can be problematic, since the same sample presented very different trends at 5 and at 10.7%. Therefore, evaluating only one concentration can lead to misinterpretations or even incomplete conclusions about the sample or about the ozone processing, as deeply discussed in our second article of the first part of this Thesis (Appendix B).

As another example, the starch gels presented distinct behaviours under the evaluated conditions (immediately after their formation and after 24 h of storage under refrigeration, at 5 and 10.7%). Consequently, we used different techniques to evaluate them – a rheometer for the recently formed gels, at Purdue University; and a texture analyser for the gel after 24 h of storage, at University of São Paulo.

The gel formation of the ozonated starches is a complex phenomenon, since it depends on a delicate balance between molecules (different sizes), electronegative groups content (carbonyl and carboxyl), starch concentration and storage conditions (time, temperature). Therefore, evaluating the gels at only one condition would not be conclusive. In fact, despite the higher amount of analyses' conditions applied in this work, if compared to others in the literature, there are still a high number of other different conditions that could be evaluated, indicating that our results are not definitive and that further studies can still be obtained. Even so, this work helped to expand the knowledge about the ozone technology, by illustrating the effects of ozonation conditions and sample's characteristics (both structure and concentration) on the obtained properties.

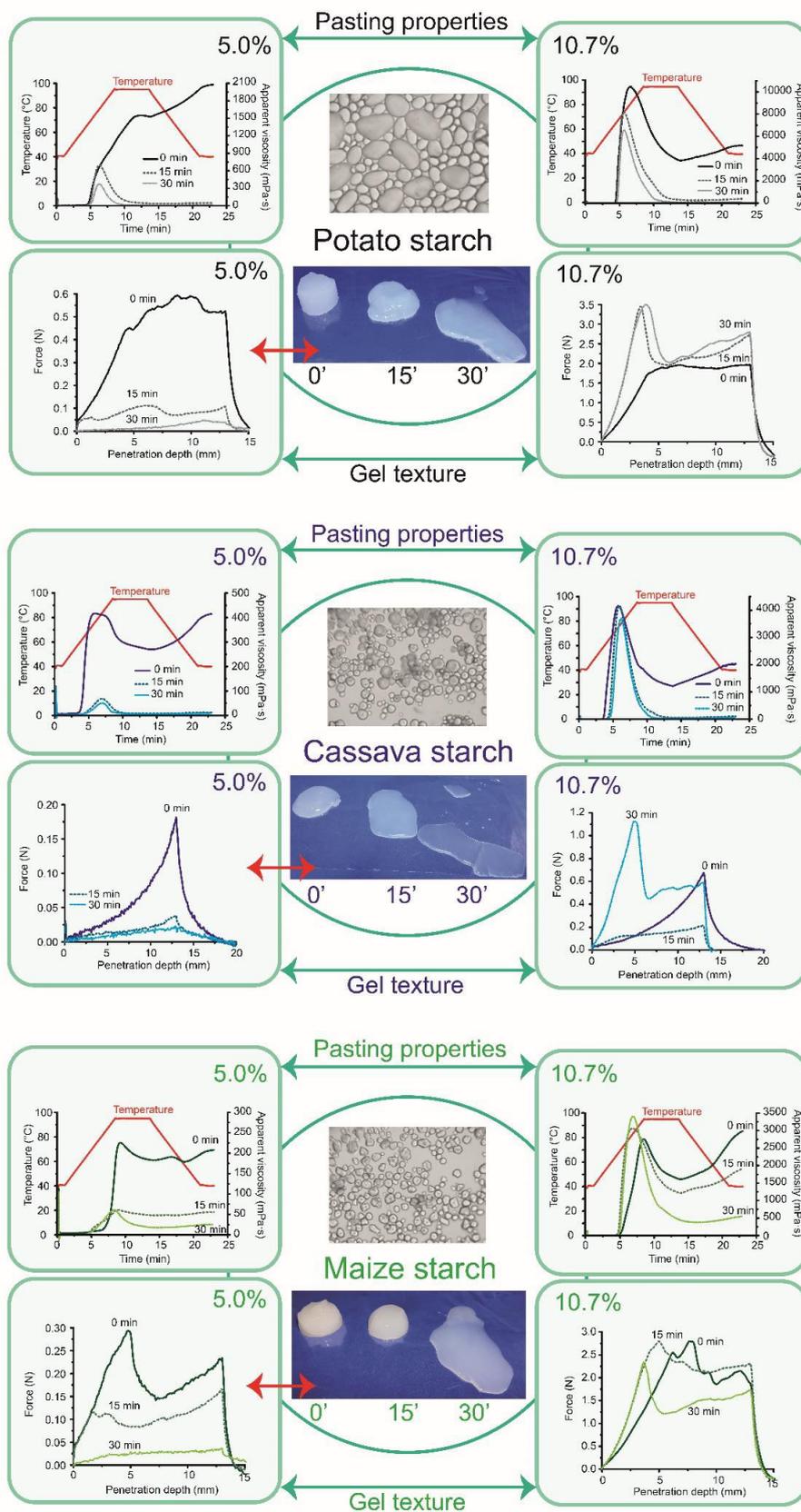


Figure 11. Main pasting and gel texture results of the cassava, maize and potato starch samples processed for different ozonation times (0, 15 and 30 min), and starch concentration (5 and 10.7%).

The obtained results were therefore described in the article “Starch modification using ozone: correlating molecular structure and gel properties in different sources”, to be soon submitted for publication, and showed in Appendix C.

Furthermore, the results obtained in this work were used as a starting point for the next two works of the second part of this Thesis. In this manner, we selected the potato starch to perform further evaluations using different analyses' settings, in order to cover other conditions not addressed in this first study, and also to propose other industrial applications for the obtained starches. Besides, we selected the maize starch to evaluate the combination of ozone and ultrasound for starch modification.

It is important to mention that the ozonated cassava starch was not selected to be further evaluated in this Thesis since its properties and possible industrial applications were already evaluated by our research group, presenting very promising results regarding its application, such as for improving the oven expansion properties of doughs [108], the 3D printability of gels [109] and to produce biodegradable films [110].

4.2.2. Expanding the possibilities of industrial applications: additional evaluations of the ozonated potato starch

In the second article of this part (Appendix D), we selected the potato starch to perform further evaluations, aiming to expand the possibilities of industrial applications. In addition, this work meant to deeply understanding the impact of the ozone technology on potato starch, by correlating the observed properties with the previously reported structural changes undergone by them [2]. For this purpose, the dynamics between temperature and water-starch interactions and its effect on the starch properties were evaluated, especially regarding its pasting properties and gel strength. Also, four different ozonation times were evaluated: 15, 30, 45 and 60 min [15].

The most promising results are related to the properties of the 15 and 30-min samples at mild gelatinization temperatures (65 and 70 °C), which presented a higher apparent viscosity (Figure 12) and a higher gel strength if compared to the control sample under the same conditions.

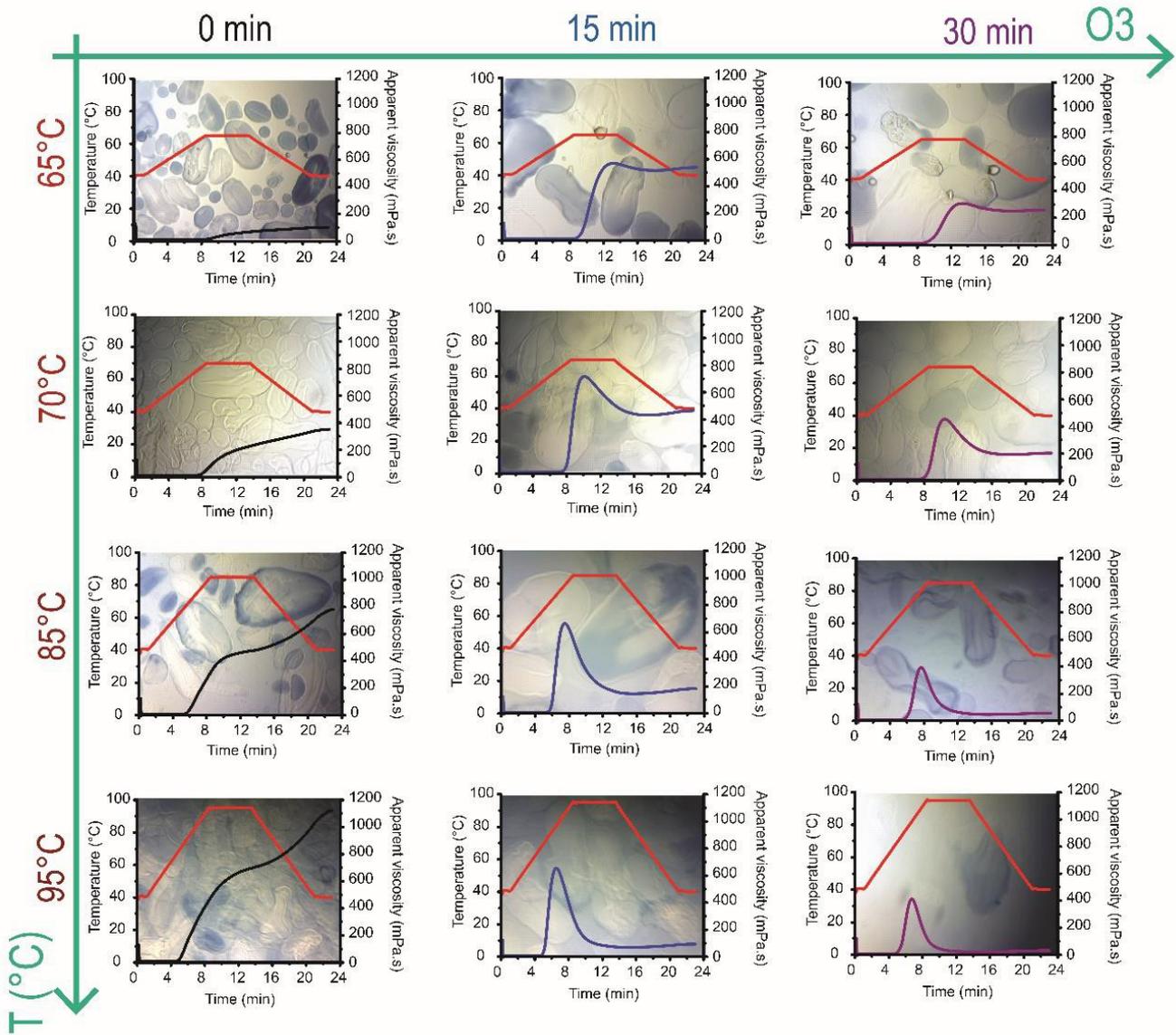


Figure 12. Pasting behaviour of the 0, 15 and 30-min ozonated potato samples gelatinized under different temperatures (65, 70, 85 and 95 °C). Behind each curve is shown their respective microscopic images, representing the appearance of their gel after the RVA analysis.

The RVA results were surprising, since the ozonated potato starch samples are usually reported to present a lower apparent viscosity if compared to their respective native samples. However, we need to emphasize that this first conclusion about the ozonated samples was based on the results obtained using standard conditions of analysis. Therefore, with our results we could prove once more that the effect of the analyses conditions on the observed properties are as important as the samples themselves. In fact, the gel texture analysis was in accordance with the RVA results, since the 15 and 30-min samples presented higher gel strength when gelatinized at 65

°C and 70 °C if compared to the other samples. The gels of the 15-min sample, obtained at 65 °C and 70 °C, were as strong as the strongest native potato starch gel (95°C), but considering 30°C of temperature difference. It is worth mentioning the industrial potential of this result.

For visually demonstrate the effect of the ozonation on the potato starch gels, a video illustrating the flow of the starch samples gelatinized at 70 °C after 24 h and after 7 days of refrigerated storage was made, being available at: <https://www.youtube.com/watch?v=WtYxreS1-78>. Also, the aspect of the gels obtained from those samples after gelatinization at different temperatures is illustrated in Figure 13.

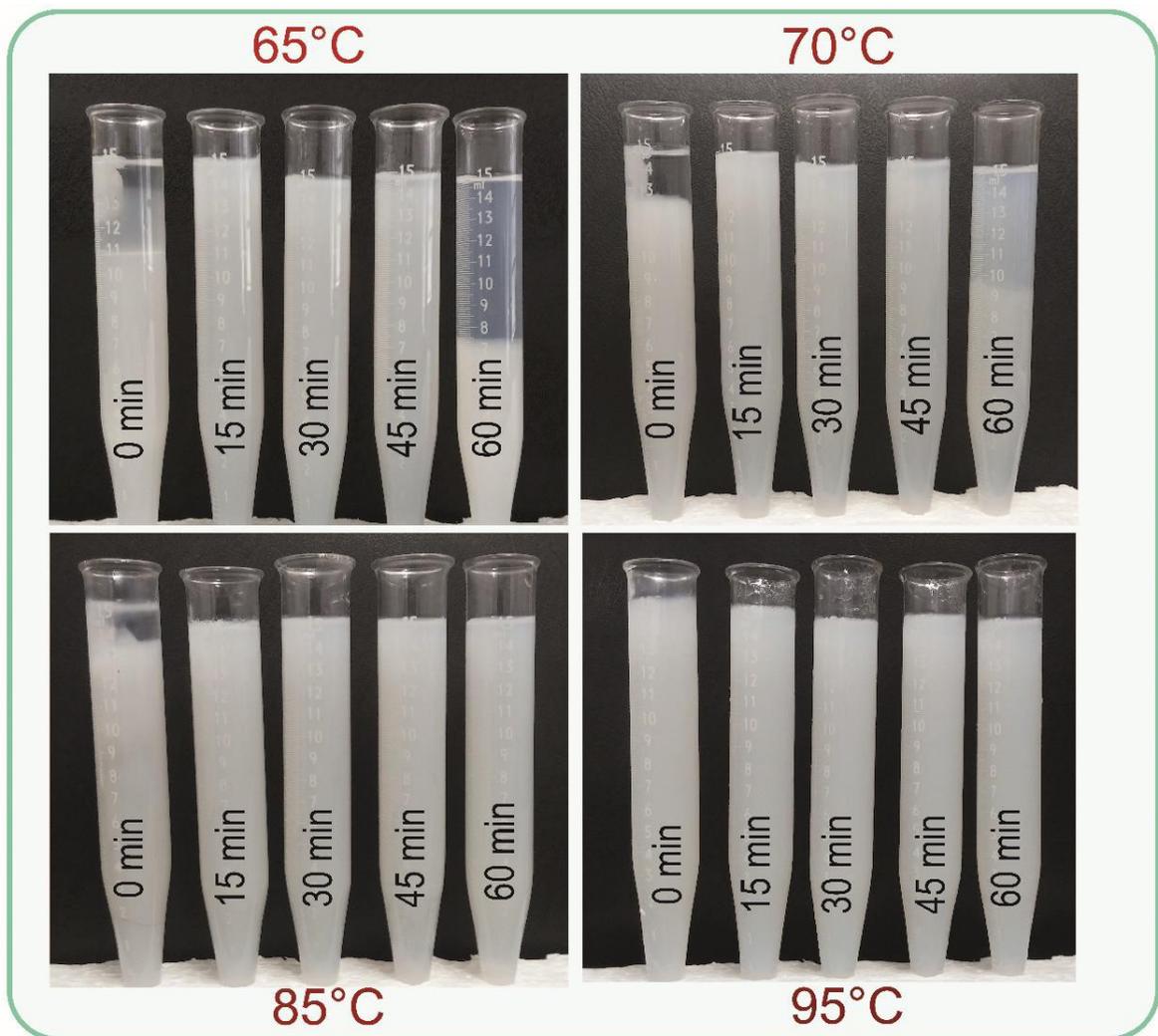


Figure 13. Aspect of the gels of the 0, 15, 30, 45 and 60-min ozonated potato starch samples after being gelatinized using different temperatures (65, 70, 85 and 95 °C).

In this work we also proposed some hypotheses to explain the observed results, correlating the easily water uptake and consequent gelatinization of the 15 and 30-min samples at 65 and 70 °C with their structure (and even illustrating our hypotheses with microscopic images). In short, the samples' properties were related to their less compact structure (due to the molecular depolymerization and the electronegative groups formation), which facilitated the gelatinization of those samples.

However, those same characteristics that facilitated the water absorption and the gelatinization of the samples at mild temperatures, favoured the granular disruption at higher temperatures (above 85 °C), decreasing their apparent viscosity. Consequently, as only the analyses performed at high gelatinization temperatures are usually reported, this remarkable characteristic of ozonated starch (i.e. the ability to form viscous pastes at temperatures ~70 °C) had never been reported before.

Therefore, the obtained results were published in the article entitled "[Properties and possible applications of ozone-modified potato starch](#)" by the Food Research International journal [15], presented in Appendix D. In conclusion, the data reported in this article expanded the comprehension of the ozone-modification process, as well as the possibility of industrial applications for the ozonated potato starch.

4.2.3. Evaluating the combination of ozone and ultrasound technologies to modify maize starch

In this work, maize starch was modified using both ozone and ultrasound technologies, each one alone and also in combination (Appendix E). The samples' molecular structure, granule characteristics and properties were evaluated [111]. A schematic representation of the ultrasound system used in this work is illustrated in Figure 14, while the ozone system was previously presented in Figure 10.

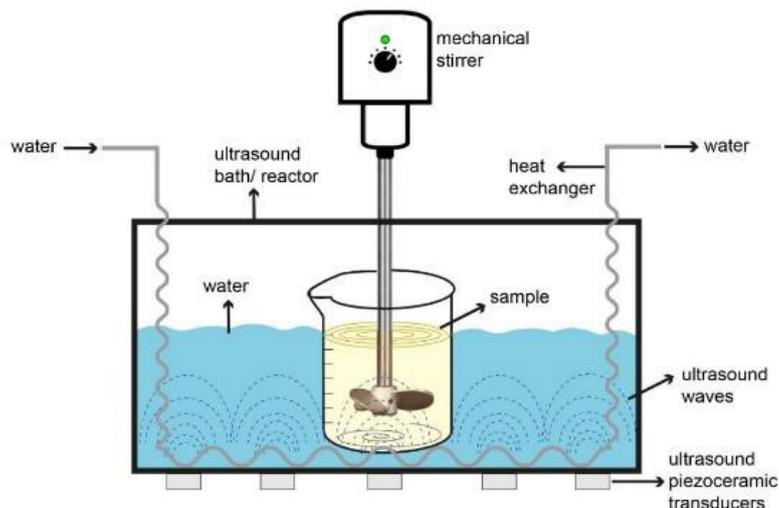


Figure 14. General aspect of the ultrasound system used for starch modification.

The ozone treatment, alone or in combination with ultrasound, led to significant changes in the starch molecules and properties at the conditions applied in this work. On the other hand, the ultrasound processing alone showed no influence on the starch's structure or properties, being this a surprising result if compared to the reported in the literature for this technology. It is important to highlight that, as discussed in the item "2. Literature Review" of this Thesis, the structural modifications of biopolymers by ultrasound treatment are reported to be highly dependent of the equipment's characteristics and the system's properties. Also, the lack of standardization during the treatments, as well as the lack of knowledge about the good practices in ultrasound processing, leads to contradictory results reported in the literature [86–88].

In any case, some evaluated properties indicated a possible effect of the ultrasound technology when it was used prior to the ozone treatment (as a higher paste clarity and a different pasting behaviour – illustrated in Figure 15), indicating that the ultrasound was able to cause changes in the samples, although not shown by the performed analyses. Based on these results, a hypothesis was raised regarding the ultrasound effect and how it apparently improved the subsequent ozone action. In short, we discussed that the ultrasound can promote changes on the starch granules (by creating cavities on their interior), that affect the way how ozone processing interacts with it. In fact, the weakening of the granules' structure induced by ultrasound treatment has been reported in several works, and it is already being used to facilitate other subsequent processes, such as to facilitate both acid and enzymatic starch

hydrolysis and to obtain porous starch granules using amylase, or starch nanoparticles [112,113].

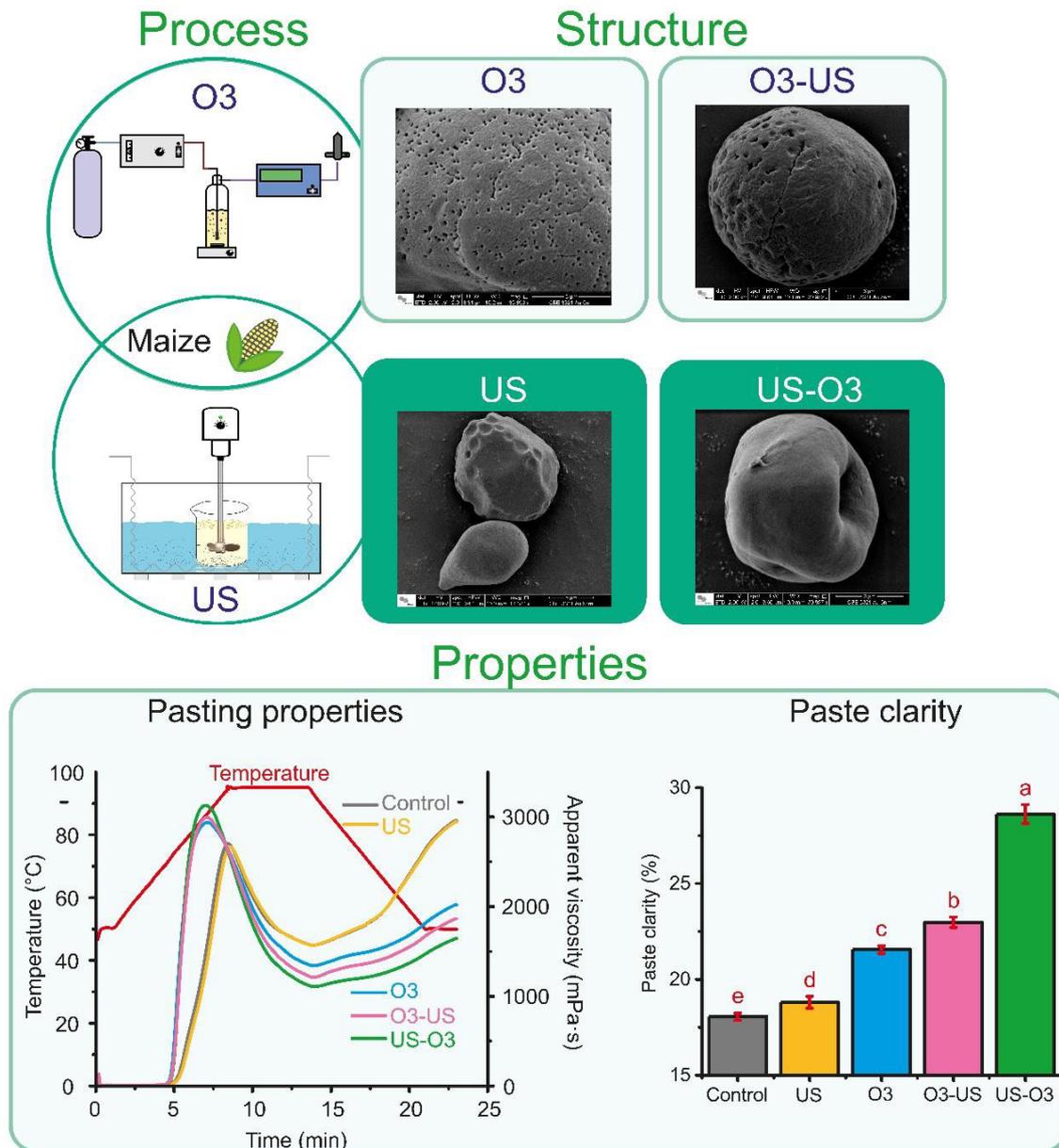


Figure 15. Schematic representation of the process, the structure and the properties (illustrated by the pasting properties and the paste clarity) of the maize starch subjected to both ozone (O₃) and ultrasound (US) modification processes, alone and also in combination (O₃-US and US-O₃).

As a conclusion, the results reported in this article indicated that the ultrasound technology, at the conditions applied in this work, was less efficient than the ozone technology to modify the maize starch. However, the ultrasound showed evidences of being able to modify the starch structure, which improved the subsequent ozone

action. Therefore, although the US technology seemed to be ineffective when used alone, it could be very useful to potentialize the ozone effect, proving to be a potential and effective alternative for starch modification.

In conclusion, the combination of ultrasound and ozone to modify starches have been little explored in the literature [89,90], and information about structural changes, as well as potential correlations between the modified starch structure and its properties, had never been reported before. Consequently, the mechanisms of the combined action of ultrasound and ozone on maize starch was firstly reported in the article entitled "[Combining ozone and ultrasound technologies to modify maize starch](#)", published by the International Journal of Biological Macromolecules [111], presented in Appendix E.

4.2.4. Using the irradiation technology to modify mung bean starch and grains

Finally, a third emerging technology was evaluated in the last article presented in this Thesis: the gamma-irradiation (Appendix F). The main objective of this work was to evaluate the efficacy of using gamma-irradiation (applying doses up to 5 kGy – which is considered high) to modify mung bean (*Vigna radiata*) starches and grains. Therefore, the effect of this technology in the mung bean grains' hydration, germination and cooking, as well as in its starch properties, were analysed and correlated with the evaluated structure [106].

Firstly, considering the irradiation effect on mung bean grains, we demonstrated that the treatment accelerated their hydration, reduced their germination capacity and improved their cooking time (softer tissues in a shorter time). It is important to mention that the only work in the literature that evaluated the effect of irradiation on the hydration kinetics of grains was performed using fava beans [114], where the authors observed an increasing hydration rate by using ionizing irradiation at doses of 2 and 5 kGy. The authors hypothesized that this behaviour was related to the increase of the starch water absorption capacity. However, contrary to the present work, this property was not evaluated in the mentioned work. On the other hand, in our work, we demonstrated that the water absorption of starch at 25 °C (hydration temperature) was not affected by irradiation, being important only at 75 °C. Therefore, by evaluating the grains further than their isolated components, but as a complex material with tissues

and cells, we proposed another explanation for the effect of irradiation on their hydration: the grain metabolism. In short, the ionizing radiation can change the cells metabolism, from membrane damage to the cell death. Therefore, the irradiated cells could lose their biological control of water transfer, letting a faster water entrance. This hypothesis can explain the hydration enhancement due to the irradiation process. In fact, the changes on the grain metabolism was also demonstrated through its germination (which was reduced).

However, as the main objective of this Thesis is related to starch modification, the structure and the properties of the irradiated starches were deeply evaluated and discussed.

In general, the results proved the efficacy of using the irradiation to desirably modify the mung bean starch. Considering the structure, the starch samples presented molecules with decreased sizes (indicating a molecular depolymerization) and lower pHs (indicating acid groups formation), being both results also usually observed for ozonated starches. Regarding the properties, the irradiation process resulted in starches with lower apparent viscosity, higher paste clarity and, in general, harder and more brittle gels (Figure 16), being those results also similar to the usually observed for ozonated starch samples. However, contrarily to the observed for ozonated starches, the irradiated mung beans did not present a lower “setback” (indicative of retrogradation) tendency. In fact, their final apparent viscosity was almost as high as their peak apparent viscosity, for all samples, although presenting decreasing values with increasing irradiation dose. All the results were related to the generation of free radicals within the starch granules, which is in fact the principle of action of this technology [98].

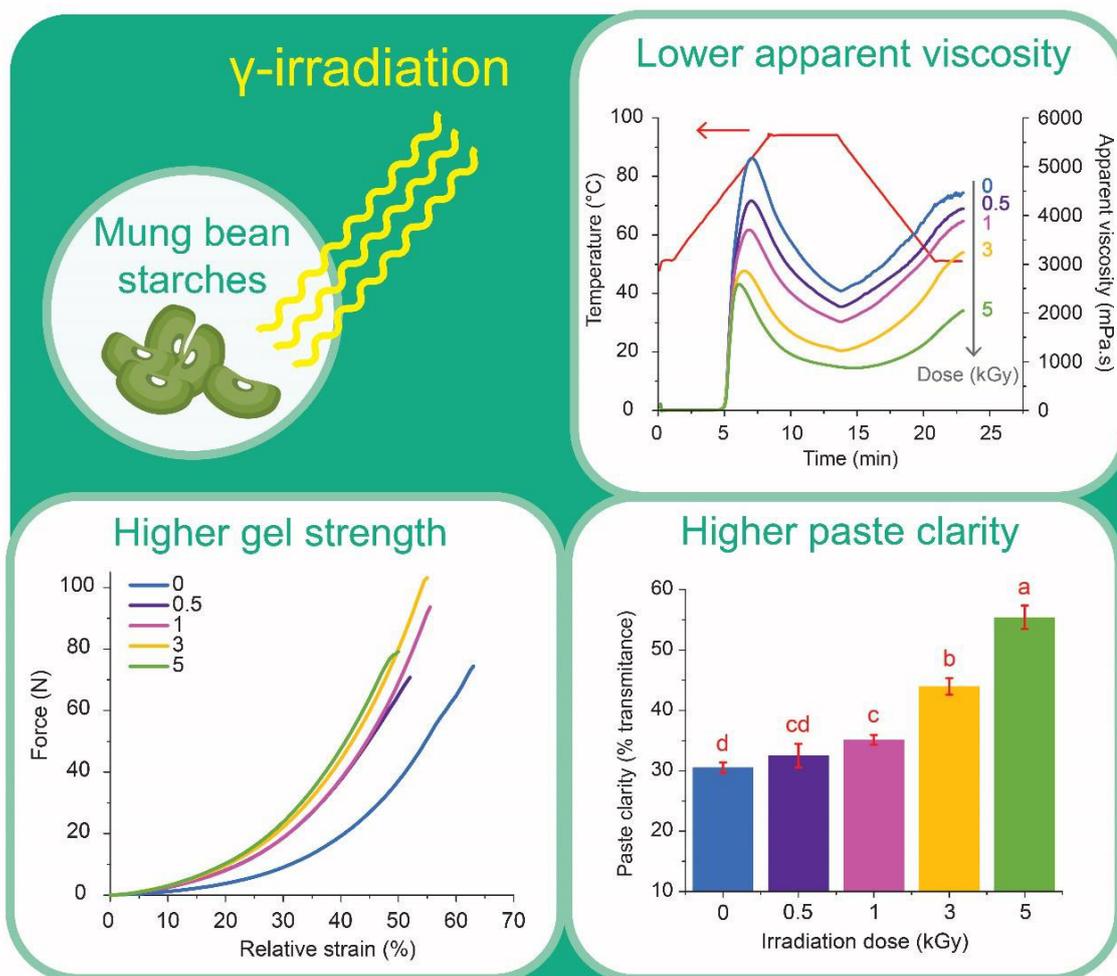


Figure 16. Schematic representation of the irradiation process on mung bean starch and the main observed properties (pasting properties, paste clarity and gel strength).

In conclusion, the results proved the efficacy of using ionizing radiation, at the doses applied in this work, to desirably modify the mung bean starch and grains, with possible industrial applications.

In fact, the effect of this type of irradiation on mung bean starch and grain was performed for the first time in this present work. Furthermore, the effect of irradiation on the properties of the mung bean grains (hydration, germination and cooking) and starches (water absorption and solubility indexes, pasting properties and paste clarity) was evaluated and correlated with their structural characteristics, resulting in an innovative and complete study. Consequently, this work was published by the International Journal of Biological Macromolecules under the title "[Irradiation of mung beans \(*Vigna radiata*\): A prospective study correlating the properties of starch and grains](#)" [106], presented in Appendix F.

4.2.5. Conclusions and future works

In conclusion of this second part of the Thesis, where different emerging technologies were used to obtain starches with enhanced properties for industrial applications, we can infer that each applied technology had its own positive and negative aspects.

Regarding the ozone technology, it proved to be a versatile and efficient technology at the conditions here evaluated. With relatively short processing time (15 min), it was possible to obtain starches with very good and applicable properties, especially if considered their gel properties at mild gelatinization temperatures (~70 °C). In fact, each processing time, combined with different starch sources, resulted in distinct functional properties, boosting the possibilities of application for the starch sources here analysed and illustrating the ozone technology versatility. Moreover, when combined with the ultrasound technology, the ozone presented a slightly improved action, especially when the ultrasound was applied first. It is important to emphasize, however, that our processing conditions were not optimized, and our treatments were always performed using excess of ozone in the reactor. Therefore, future studies evaluating the scale up and the processing optimization could be carried out.

On the other hand, the ultrasound technology presented no measurable effect on the maize starch when evaluated alone, at the conditions applied in this work. However, when this technology was combined to the ozone process, an improved effect of the last was observed. Therefore, as future studies, the first suggestion would be evaluating another ultrasound system, maybe a probe instead of an ultrasonic bath (although the scale up of ultrasonic probes is a problem to be considered). Another possibility would be evaluating different processing conditions and starch sources, aiming to better understand the ultrasound phenomena and how it acts on the starch granules, in order to optimize the process. It is important to mention, however, that the good processing practices must be taken into consideration, such as temperature control or precautions with the probe wear (with the possibility of metallic particles detaching) – which is not always carried out in the literature.

Finally, considering the irradiation technology, it proved to be an efficient and highly penetrating process, resulting in applicable properties for the starch samples. Furthermore, the processing was simple, and the samples' preparation was minimal.

The main issues about this technology are related to the radioisotopes (^{60}Co in this case) obtaining and correct disposal. Moreover, the consumer's biased opinion against it impairs its utilization, especially regarding the food industry. Therefore, as future works, we suggest mapping the main opinions and concerns of the consumers, in order to evaluate how and if this technology could be applied.

As a general recommendation of future studies for the three technologies, an evaluation of the overall costs related to the use of each one would be of great value, in order to facilitate their industrial possible application. Also, the evaluation of the interaction of the obtained samples with other ingredients in the food industry, such as proteins, lipids, or even with food additives, would be of great value for further applications.

5. GENERAL CONCLUSIONS

The first main conclusion of this Thesis was that finding useful functional properties for industrial application from novel starch sources, especially in their native form, is quite challenging. In fact, the high number of uncontrollable factors, such as low process yield, a complicated starch extraction, and the struggle in finding new starch sources with potential to be commercialized complicates even more this task. However, there are some promising alternative starch-rich sources that are still underutilized, as the Andean arracacha root, which naturally presents valuable properties and can bring a more “natural” claim to processed foods. In fact, in this Thesis the arracacha starch was characterized and possible applications for this starch sample were proposed.

Another possibility to get advantage of non-modified starches is by varying the conditions in which they are applied/ processed, which was also evaluated in the present Thesis. By changing the concentration and gelatinization temperature, for example, different results were obtained – being also different from the expected ones.

However, the native starches' properties are still limited, and changing the starch concentration in a product could bring undesirable consequences. Therefore, despite changing the processing conditions would be a simple way to solve some industrial issues regarding starchy products, the starch modification is still necessary in some cases.

Considering the emerging technologies used for starch modification in this Thesis, some important conclusions were made. The first one is that each technology has its particularities and affects differently the starch sources, thus resulting in distinct properties. The ozone, for example, is highly oxidative and changes both molecular sizes and hydroxyl groups of the starch samples. The ultrasound can mechanically affect the starch granules. And the irradiation produces free radicals within the interior of the starch granules. Furthermore, not only the process or processing conditions, but also the starch source (structure) and how it will be applied in the desired product will influence the obtained properties. Moreover, each technology has its own needs regarding the facilities, costs and sample preparation, among other particularities. Therefore, the selection of the best or more suitable technology depends on several factors, and each case needs to be deeply studied.

6. FUTURE STUDIES

Considering the main results and discussions presented in this Thesis, some perspectives for future works can be suggested.

Regarding the non-modified starches, further characterization of novel botanical sources would be valuable. To that end, many approaches are valid, such as: exploring underutilized starch-rich sources, exploring starch sources with potential to present desirable functional properties or exploring starches extracted from agro-industrial wastes (in order to give a better destination for this material, thus making them as by-products or even co-products).

Another possibility for future works using non-modified starches would be to better evaluate and understand the behaviour of different starch sources under non-standard conditions of analyses, correlating the obtained properties with their structure and thus optimizing their industrial applications. Also, modelling starch samples' behaviour under the most varied conditions would be valuable to avoid using empirical assessment for each starch application.

On the other hand, considering the modified starches and the applied modification processes, the possibilities for future works regarding the ozone technology are related to the process' optimization and scale up, facilitating further industrial applications. Also, deep evaluations of the obtained modified starches, as well as the modification of unusual starch sources using ozone, are still of great relevance.

Considering the ultrasound technology, the evaluation of different ultrasound systems, as well as its positive and negative aspects regarding optimization and scale up, is crucial. Also, definitive analyses proving the real effect of the ultrasound technology on the starch granules are still necessary.

Finally, considering the irradiation technology, which has two main inherent disadvantages (the obtaining and correct disposal of the radioisotopes and the consumer's biased opinion against it), as future works we suggest studying disposal options for the cobalt and also to map the main opinions and concerns of the consumers, in order to evaluate how and if this technology could be applied. Further, using accelerated electrons as irradiation source would avoid the needs for radioisotopes.

Also, as a general recommendation of future studies, an evaluation of the overall costs and environmental impact related to each approach or technology is always of great value to estimate an industrial feasibility. Further analyses, as the interaction of those obtained starches with food ingredients or additives, as well as a sensory evaluation of them, would be of great value for further food applications.

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APPENDIX

APPENDIX A: Structure and properties of starches from arracacha (*Arracacia xanthorrhiza*) roots

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Structure and properties of starches from Arracacha (*Arracacia xanthorrhiza*) roots

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ABSTRACT

Arracacha (*Arracacia xanthorrhiza* Bancroft) is an underexplored Andean root with a high starch content. In this work, starches from two different varieties of Peruvian arracacha were evaluated and characterized in relation to their granule morphology, molecular structure and properties. The starches presented round or polygonal shapes, with a mean diameter of ~20 μm and B-type granules. They were rich in amylopectin molecules with long chain lengths (with the ability to complex iodine) and some with intermediate sizes (indicating a defective crystalline structure). The starches presented low gelatinization temperature, enthalpy of gelatinization and tendency to retrogradation and high peak apparent viscosity and swelling capacity, even at moderate temperatures (60 °C), characteristics of high interest for industrial purposes. Besides, the starches presented a smooth and elastic gel and a high paste clarity. Overall, the arracacha roots presented attractive properties and can be used as an alternative botanical source for starch extraction.

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1. Introduction

Starches are biomolecules with many industrial applications and relevant economic importance. They are used in many industries, for food (human and animal), pharmaceuticals, cosmetics, chemicals, petrochemicals and textiles, among others. Consequently, different properties are demanded, highlighting the importance of characterizing new natural sources.

Arracacia Bancroft is a genus of about 30 species, *Arracacia xanthorrhiza* being the only one cultivated [1]. The *Arracacia xanthorrhiza* Bancroft is possibly one of the oldest cultivated plants in the Andes, earlier even than the domestication of the potato (*Solanum tuberosum*) [2, 3]. The name “arracacha” is derived from the Quechua word “racacha” [4], and has been accepted as a standard term in several literatures, including in English. According to Hermann [1], other terms for the arracacha, such as the “Peruvian carrot” or “Peruvian parsnip”, should be avoided to prevent misunderstandings. Currently, this root is cultivated in areas from Mexico to South America [1].

If compared to other popular root crops, like cassava and carrots, arracacha can be considered high perishable, a characteristic that constrains its commercial exploitation *in natura* [5]. In contrast to its current low consumption, commercialization and industrialization, which make this “underutilized”, the arracacha is an Andean crop adapted to

a wide climatic range in Peru [6], if compared with high-altitude species such as oca, olluco, maca or mashua, with a narrow ecological range [1]. Consequently, industrial applications for this root are highly relevant in order to boost its commercial importance and thus allow its cultivation.

An alternative for the best use of arracacha would be for starch extraction, since this root has a high starch content that is relatively simple to extract. In fact, there are some works with very promising results regarding the content and characterization of starch from arracacha varieties from Brazil [7–11], Venezuela [12] and Colombia [13]. However, although the Peruvian germplasm bank is very broad regarding the arracacha [14], as far as we know, no studies have been published about starches from the Peruvian varieties. Starch characteristics, such as granule and molecular size distribution, shape and composition, are largely influenced by its botanical origin [15], leading to different properties and possible applications.

Consequently, this work aims to characterize and evaluate starches from two different varieties of Peruvian arracacha (“arracacha morada” and “arracacha amarilla”), describing their structure, properties and possible applications.

2. Material and methods

2.1. Material

The starches were extracted from arracacha roots (*Arracacia xanthorrhiza* Bancroft), purchased from farmers in Peru. Two different

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varieties were evaluated in the present work: “arracacha morada” (“morada” = purple) and “arracacha amarilla” (“amarilla” = yellow). The roots were collected and transported to the “Universidad Nacional Agraria La Molina” in Lima (Peru), and starch extraction was performed immediately.

The arracacha morada was cropped in Huaraz (Peru) and the arracacha amarilla was harvested in two different parts of the Huánuco region (“arracacha amarilla A” = valley region; “arracacha amarilla B” = high altitude region). It is important to mention that the effect of the planting site on the starch properties was not focus of the present study.

Furthermore, starches from other sources were used for comparison: potato starch (extracted, characterized as described by Castanha et al. [16]), maize starch (Argo CS 3400), high-amylose maize starch (i.e., a starch source with ~72% of amylose - Hylon VII), waxy maize starch (i.e., a starch source with ~98% of amylopectin - Amisol 4000) and cassava starch (kindly provided by “Ingredion Brasil Ingredientes Ltda”). The purpose of analysing these samples, in the same conditions of analysis used for the arracacha starches, was to provide data from common and widely known commercial starches, allowing a direct correlation among their properties.

All the chemicals were of analytical grade and used without further purification.

2.2. Starch extraction

The arracacha roots (composition shown in Table S1, supplementary material) were washed and cut into slices approximately 5 cm thick and immediately crushed in a laboratory scale blender for 3 min. The starch extraction was then performed according to the process described by Castanha et al. [16]. The starch obtained was dried in an air circulation oven at 35 °C to a moisture content of approximately 13%. The dried starch was ground in a mortar and sieved (250 µm) for further analysis. The proximal composition of the obtained starch is shown in Table S1 (supplementary material).

2.3. Roots and starch chemical composition

The protein and ash contents from the arracacha roots and starches were analysed using the methods described in AOAC [17]: the nitrogen content (for the crude protein content) was determined by the micro-Kjeldahl method using factor 6.25, and the ash content was determined in a muffle furnace at 550 °C until complete calcination.

The starch content of the roots was determined using a total starch determination kit (Code K-TSTA, Megazyme, Ireland), consisting of the enzymatic (α -amylase/amyloglucosidase) breakdown of the starch and subsequent colorimetric evaluation of the remaining glucose. This kit is based on the AOAC 996.11 [17] and AACC 76-13.01 [18] methods.

The mineral content was determined using an energy dispersive X-ray fluorescence spectrometer (Shimadzu EDX-720, Japan), according to Tezotto et al. [19].

The pH of a 10% (m/m) starch slurry was determined in a calibrated potentiometer (Tecnal, TEC-5 mode, Piracicaba, Brazil), under constant stirring, according to Adolfo Lutz Institute [20].

2.4. Starch characterization

2.4.1. Granule morphology: particle size distribution, scanning electron microscopy (SEM), light microscopy and polarized light microscopy

The size distribution of the starch granules (particles) was determined using a Laser Analyser (Partica LA-950V2 Laser Particle Size Analyser HORIBA, Japan) and the LA-950 software for Windows (HORIBA, Japan). The samples were dispersed in ethanol (99.5%). The volume-based mean diameter ($D[3, 4]$, Eq. (1)) and the area-based mean diameter ($D[2, 3]$, Eq. (2)) were evaluated, as was the distribution. Both area and volume equivalent diameters were evaluated since

the $D[3, 4]$ is more influenced by large particles, whereas the $D[2, 3]$ is influenced by the smaller ones [21, 22].

$$D[4, 3] = \frac{\sum_i n_i d_i^4}{\sum_i n_i d_i^3} \quad (1)$$

$$D[3, 2] = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \quad (2)$$

Scanning electron microscopy (LEO 435 VP, Leo Electron Microscopy Ltd., Cambridge, England) was used to evaluate the starch granule morphology and general appearance. The microscope was operated at an acceleration voltage of 20 kV. Dry starch was sprinkled onto double-sided adhesive tape placed in the middle of circular stubs. The stubs were coated with a 30-nm gold layer and then evaluated in the microscope.

A light microscope (model L1000, Bioval, Curitiba, Brazil) with a 20-W halogen lamp was used to evaluate the shape and surface of the starch granules. A 1% dispersion of starch in distilled water was prepared and immediately observed in the microscope. To better distinguish the granules, a drop of Lugol solution (I_2 and KI in ethanol) was mixed with a drop of the starch dispersion onto the glass slide, which was covered by a glass cover slip. The magnification used was 400 \times and a 1.3-megapixel portable camera was used to obtain the images. To observe the Maltese crosses of the starch samples, a polarized light filter was coupled to the same microscope, and the same starch dispersion was used but without the Lugol solution.

2.4.2. Molecular size distribution

The molecular size distribution profile of the starch molecules was determined by gel permeation chromatography (GPC), according to Song and Jane [23], with small modifications. A glass column (2.6 cm diameter and 70 cm high) packed with Sepharose CL-2B gel (Sigma, Sweden) was used. 0.1 g of starch was mixed with 10 mL of Dimethylsulfoxide (DMSO; 90%, Labsynth, Brazil) and heated in a bath of boiling water for 1 h and then kept at 25 °C for 24 h under constant stirring. An aliquot of 3 mL of this solution was mixed with 10 mL of absolute ethanol and then centrifuged for 30 min at 3000 g. The precipitated starch was dissolved in 9 mL of boiling distilled water and placed in a bath of boiling water for 30 min. An aliquot of 4 mL was then upwardly eluted in the chromatographic column, with an eluent solution (25 mmol·L⁻¹ of NaCl and 1 mmol·L⁻¹ of NaOH), at a rate of 60 mL·h⁻¹. A fraction collector (Gilson, model FC203B, Middleton, England) was used to separate the sample into 4-mL portions, and these were then analysed for total carbohydrate content at 490 nm by the phenol-sulfuric [24] and blue value at 620 nm [25] methods, using a microplate reader (Asys Expert plus, Biochron, England). A glucose sample was used as a marker to indicate the end of the analysis.

2.4.3. Apparent amylose content

The apparent amylose content was determined according to the ISO methodology [26]. A reference curve was firstly made using standard amylopectin (A8515) and amylose (A0512 type III) from Sigma-Aldrich Pty Ltd. (Castle Hill, NSW, Australia). Starch grains were dispersed in ethanol 95% and NaOH 1 mol·L⁻¹, and then gelatinized in a boiling water bath for 10 min. An aliquot of 18 mL was mixed with 2 mL of NaOH 0.09 mol·L⁻¹. Then, an aliquot of 5 mL of this solution was transferred to a volumetric flask of 100 mL with 1 mol·L⁻¹ acetic acid and iodine solution (0.2% I_2 , 2% KI), forming a complex of blue colour, which was quantified by spectrophotometry at a wavelength of 620 nm (spectrometer Femto, Model 600S, São Paulo, Brazil).

2.4.4. X-ray diffraction patterns

The starch samples were maintained in a desiccator containing saturated BaCl₂ solution (25 °C, a_w = 0.900) for 10 days, to ensure a constant activity of water. An X-ray diffractometer (Shimadzu XRD 7000, Tokyo, Japan) with copper radiation was used at an angle 2θ ranging from 3 to 40°, using the following working conditions: scan rate of 2°·min⁻¹, 40 kV and 30 mA. The curves obtained were smoothed using the Origin software, version 9.1 (Microcal Inc., Northampton, MA, USA).

2.4.5. Thermal properties

The thermal properties (gelatinization and retrogradation) were evaluated using a Differential Scanning Calorimeter (DSC 2010, TA Instruments, New Castle, DE, USA), operating with the Thermal Advantage V 1.1A software (1999, TA Instruments). The Universal Analysis 2000 V 4.2E (TA Instruments) software was used to analyse the data. For the gelatinization study, 3 mg of starch (dry basis) was mixed with 7 μL of deionized water and placed in a hermetically sealed aluminium pan. The samples were kept at room temperature for 1 h before the measurement. The scanning temperature was from 30 °C to 100 °C and the heating rate was 10 °C·min⁻¹. An empty pan was used as the reference. The onset, peak and conclusion temperatures and enthalpy of gelatinization (ΔH), on a dry basis, were obtained. In sequence, the pans with the gelatinized starch samples were stored for 7 days at 5 ± 2 °C for the retrogradation studies. Then, the same protocol mentioned above was used to evaluate the thermal properties after retrogradation.

2.4.6. Pasting properties

Rapid Visco Analyser equipment (RVA-4, Newport Scientific Pvt. Ltd., Australia) using the ThermoLine for Windows software (version 3.0) was used to determine the pasting properties of the starch samples. A suspension of 3 g (14% moisture basis) of starch in 25 g of distilled water was homogenized (for 10 s at 960 RPM) and then analysed under a constant shear (160 RPM), under 2 different heating protocols. In the first one, the suspension was initially held at 50 °C for 1 min, then heated to 95 °C at a rate of 6 °C·min⁻¹, then kept at 95 °C for 5 min, followed by cooling to 50 °C at a rate of 6 °C·min⁻¹, and finally holding it at 50 °C for 2 min. In the second protocol, the suspension was first held at 30 °C for 1 min, then heated to 95 °C at a rate of 9 °C·min⁻¹, then kept at 95 °C for 5 min, followed by cooling to 30 °C at a rate of 9 °C·min⁻¹, and finally holding it at 30 °C for 2 min.

2.4.7. Gel firmness

The firmness of the starch gel was determined by instrumental texture, using a Texture Analyser (TA.XT Plus, Stable Micro Systems Ltd., Surrey, UK) with a load cell of 50 kgf (490.3 N). After the RVA analysis, the gels obtained were stored in plastic cups (40 mm diameter × 20 mm height) for 24 h at 5 ± 2 °C before texture evaluation. To ensure uniform moisture of the samples and avoid drying, they were kept in a desiccator with water at the bottom. The samples were penetrated using a 0.5-cm cylindrical probe (P/0.5R) at 1 mm·s⁻¹. The force measured by the equipment as a function of the penetration depth was then used to evaluate the gel firmness.

2.4.8. Water absorption index (WAI) and water solubility index (WSI)

The water absorption and solubility indexes were evaluated according to the procedure described by Anderson et al. [27], with minor modifications. 0.5 g of starch (dry basis) and 6 mL of distilled water were mixed in pre-weighed centrifuge tubes. The tubes were then placed in a thermal bath with stirring for 30 min at different temperatures (30, 40, 50, 55 and 60 °C) and then centrifuged at 3000 g for 10 min. The supernatant was dried and weighed (DS), and the precipitated retained in the tube was also weighed (PT). The WAI (Eq. (3)) and the WSI (Eq. (4))

were then calculated considering MS as the mass of the sample, on a dry basis.

$$WAI \left(\frac{\text{g water}}{\text{g starch}} \right) = \frac{PT - (MS - DS)}{(MS - DS)} \quad (3)$$

$$WSI (\%) = \frac{DS}{MS} \cdot 100 \quad (4)$$

2.4.9. Paste clarity

The paste clarity of the starches was evaluated by measuring the transmittance (T%) of the samples as described by Craig et al. [28] and modified by Aplevicz and Demiate [29]. 0.2 g of starch was mixed with 20 mL of distilled water in test tubes with screw caps. The tubes were then placed in a thermal bath with boiling water for 30 min and stirred individually every 5 min. The tubes were then cooled to room temperature and evaluated in a spectrometer at a 650 nm wavelength (Femto, Model 600S, São Paulo, Brazil).

2.5. Experimental design

The starch samples were extracted from one batch of arracacha (for both the starches and roots characterized in this work, Table S1, supplementary material). Each analysis was repeated at least twice, and each repetition was done at least in duplicate.

3. Results and discussions

3.1. Arracacha starch structure

3.1.1. Roots and starch proximal composition

Both the proximal composition (Table S1) and mineral content (Table S2) of the arracacha roots and their respective starches are provided in the supplementary material.

Considering the arracacha roots, it is possible to observe that they are a rich source of starch (more than 50% of the dry material).

The arracacha starch phosphorus content (~200–270 mg·kg⁻¹) is relatively low if compared to the potato (*Solanum tuberosum*) starch, a rich source with a phosphorus content of approximately 800 mg·kg⁻¹ [16]. However, Hoover [30] compared the organic phosphorus content reported in different works, and the arracacha starch phosphorus content is higher than the content in the starches from 12 other roots and tubers evaluated (except the potato). It is important to highlight that phosphorus is a component that, in general, increases paste viscosity and paste lightness and decreases retrogradation rates of starches [30, 31].

3.1.2. Morphology of the granules

The arracacha starch particle size distribution is presented in Fig. 1.

In general, the arracacha starches granules was around 20 μm (Fig. 1-C). If compared to starches from different sources (Fig. 1-D), the distribution of the size of the arracacha is similar to that observed for cassava starch. Maize starch has a higher granular population around 30 μm, while, with larger granules, most of those in potato starch appear in the 50 μm range. The volume-based mean particle diameter of the arracacha morada sample is higher than the one observed in the arracacha amarilla starches (Fig. 1-B), indicating that the diameter of the granules in this sample is larger than those of the arracacha amarilla.

Fig. 2 shows the general aspect of the arracacha roots and their respective starch granules under different views (light microscopy, polarized light microscopy and scanning electron microscopy). A complementary figure, containing only the SEM images of the starch samples, is available in the Supplementary material (Fig. S1).

Considering the morphology of the starch granules (Fig. 2), in general, they are round-shaped, with smaller ones with irregular

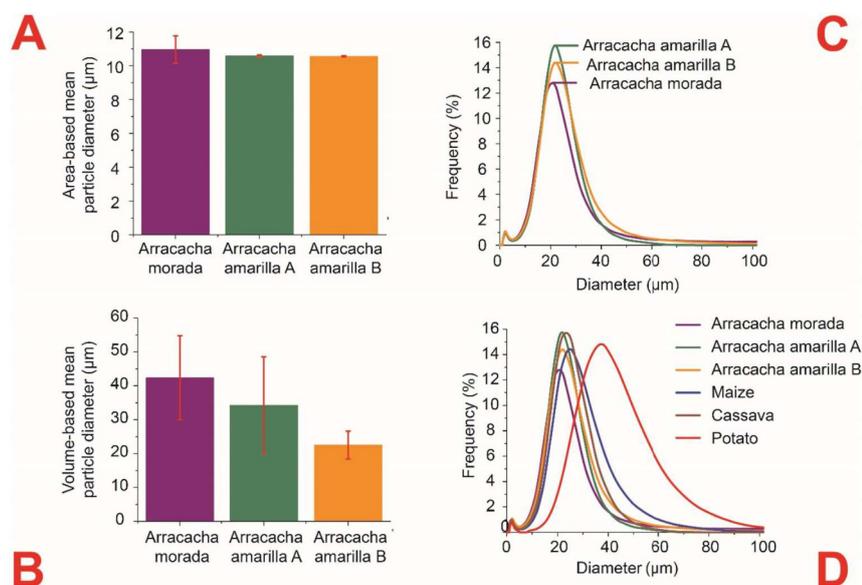


Fig. 1. Particle size distribution of the arracacha starches. (A) Area-based mean particle diameter; (B) volume-based mean particle diameter; (C) size distribution of the arracacha starches; (D) comparison among the size distribution of arracacha starches and starches from maize, cassava and potato starches. Vertical red bars represent the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(polygonal) shapes, the granules of the morada variety being the most irregular. Besides, some granules in all samples had cracks and fissures on their surfaces, probably due to being closely packed in the cells of the arracacha roots in a “Lego-like” conformation, as proposed and demonstrated for arracacha starches from Colombia by Londoño-Restrepo et al. [13] (this compactness of the granules can be better observed in the electron microscopy images in Fig. 2 and in Fig. S1).

In fact, Alonso-Gomez et al. [32] presented SEM images of native cassava starches inside the cells and isolated, and it was possible to observe that the starch granules were “compressed”, presenting a similar format to those observed in this work.

Furthermore, while studying arracacha starch from Brazilian roots (*Arracacia xanthorrhiza*, “Amarela de Senador Amaral” variety), Rocha et al. [11] also observed round and irregular-shaped granules, with a tendency to separate and crack, and some depressions on their surfaces - being consistent with the Peruvian roots.

Pérez et al. [33] argued that, on a macromolecular scale, the surface of starches can differ significantly between different botanical sources. The authors also commented that channels and pores can be found in starch granules affected by plant metabolic activities, such as its growth. This can be the case here studied.

3.1.3. X-ray diffraction

The X-ray diffraction patterns of the arracacha starches are presented in Fig. 3-E.

Starches were proved to be a crystalline material by P. Scherrer, almost 100 years ago [34]. Since then, studies regarding the starch crystallinity have been performed using the X-ray analysis. Several of these studies classifies the X-ray diffraction patterns of different starch samples into 3 different types: “A”, “B” or “C” [34, 35]. According to this classification, the three varieties of arracacha starch can be classified as presenting a B-type pattern, typical of tubers and roots [34].

However, recent studies [13, 32, 36, 37] have been using a new approach to analyse the X-ray patterns of different starch samples, which are based on the comparison of the obtained patterns with standards from a database of Powder Diffraction Files (PDF-4) [38]. According to this approach, the X-ray diffraction patterns must be compared to

standard patterns of “pure” amylose and amylopectin samples and with the peaks based on the α -amylose database (PDF 43–1858). It worth be mentioned that the amylopectin PDF database is still under study, as well as the amylose source used as reference is different from ours [32, 37].

In this way, when comparing our results with the amylose and amylopectin diffraction patterns obtained by Londoño-Restrepo et al. [37], we can observe some interesting results. In the arracacha samples, the peaks located at $\sim 17^\circ$, 20° , 22.5° and 24° are correspondent to the amylose crystalline structure, while the $\sim 5.5^\circ$ peak was not observed in the PDF database. Alonso-Gomez et al. [32] believe that the 5.5° peak corresponds to nanocrystals.

However, despite using standards, the comparisons and the conclusions must be carefully evaluated, since the moisture content and the amount and conditions of the amylose and amylopectin molecules greatly influence the X-ray patterns [35]. In fact, as precisely stated by Londoño-Restrepo et al. [13], the crystalline structure of starches are far from been completely understood, and requires further studies.

3.1.4. Molecular size distribution and apparent amylose content

The apparent amylose content and the molecular size distribution of the arracacha starches are shown in Fig. 3.

The apparent amylose content obtained was high for starches of arracacha morada ($39.0 \pm 1.5\%$), amarilla A ($35.8 \pm 0.7\%$) and amarilla B ($35.7 \pm 0.7\%$). Works in the literature report much smaller amylose contents than those obtained in this work, with values from 18 to 20% from the Brazilian [9, 11] and Venezuelan [12] varieties.

However, the limitations of the analytical method and the interpretation of the results must be highlighted once again.

The amylose content obtained by this colorimetric analysis with iodine is considered “apparent”, since long chains of amylopectin can also develop the characteristic blue colour of the amylose-iodine complex, leading to an “overestimated” amylose value [39–41]. Jane et al. [39] compared the absolute and apparent amylose values obtained for different starch sources and observed that, in some cases, the difference could be over 20%. This overestimation took place especially for the B-

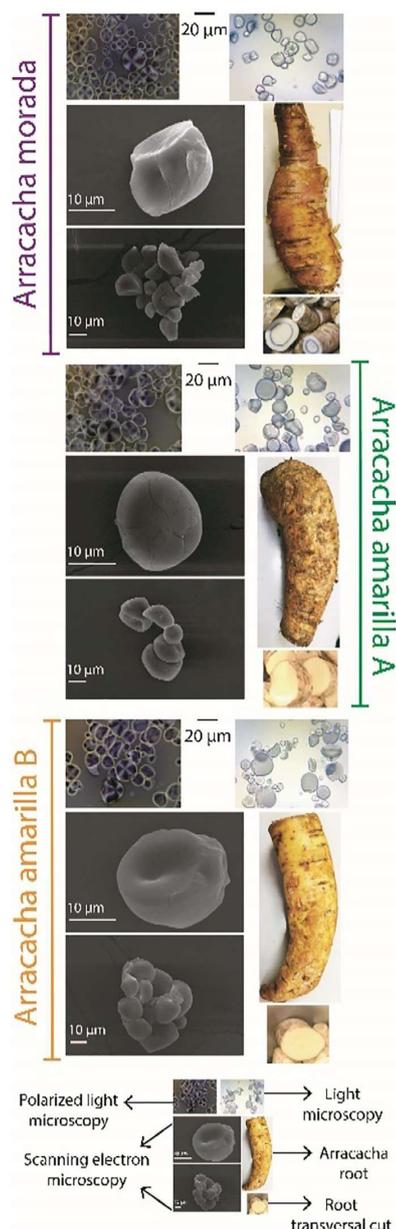


Fig. 2. General aspect of the arracacha roots and their respective starches.

type starches - as is the case of arracacha starches, as shown in Fig. 3-E. In fact, Rocha et al. [11] observed a high amylopectin-iodine affinity while studying starch from a Brazilian arracacha, concluding that the amylopectin in the samples could contribute to the blue colour observed in the apparent amylose analysis.

In such a way, the amylose content based on the iodine complex could lead to a false interpretation of the amylose value. So, a gel permeation chromatography (GPC) analysis was performed to elucidate better the proportion and chain length of both amylose and amylopectin in the arracacha starches.

The GPC analysis consists basically of the separation of the starch fractions based on their molecular sizes. Thus, the molecules with higher chain lengths elute first, since they are not able to penetrate the gel pores. On the other hand, the lower chain-length molecules are retained in the column and elute later [42].

Normally, the amylopectin molecules are described as being large and highly branched [42]. Therefore, they are supposed to be mainly contained in the first fraction, forming the first chromatogram peak, while the following portions are normally related to the amylose molecules. However, the starch molecules cannot be simply divided into only two fractions, since a number of small molecules of amylopectin may elute in the later fraction, or long chains of amylose may elute in the first. Therefore, the amylose and the amylopectin fractions are not sharply separated, but blend into each other through intermediate fractions [41, 42].

Considering this, the arracacha starches were evaluated using two different techniques in the GPC: the total carbohydrate method (based on a phenol-sulfuric colorimetric method) and the blue value method (also based on the starch-iodine complex). Once more, maize, high amylose and waxy commercial starches were used for comparison purposes.

Analysing the results (Fig. 3-A and -B), it is possible to observe that in some cases the amylopectin molecules can form the blue complex with iodine, as discussed by Jane et al. [39]. For example, while analysing the results of the blue value (Fig. 3-B), it is clear that waxy starch (which presents about 98% of amylopectin, thus should not form an intense blue colour with iodine) presented a distinct peak in the “amylopectin elution area”, showing that these molecules were coloured by the iodine in the analysis. Similarly, when analysing the results of the blue value for the arracacha starches, the first peak is more pronounced when compared to the first peak in the other samples, indicating high chain-length molecules (probably amylopectin) that can form the iodine complex. In fact, when comparing the graphs of the total carbohydrate (Fig. 3-A) and blue value (Fig. 3-B), it is possible to observe that the arracacha starches do not follow the same patterns as maize starch, for example. A first peak in the total carbohydrate analysis for maize starch was higher than the one observed for the arracacha starches, but lower in the blue value analysis, confirming the theory that the amylopectin chains of the arracacha starches are being complexed with iodine.

Another important aspect that can be observed in the GPC results is the presence of a “shoulder” after the first peak in the chromatogram of the total carbohydrate results (Fig. 3-A), which is not present with maize starch (with a thin and well-delimited first peak). This can indicate a proportion of molecules (probably amylopectin) with “intermediate” sizes, suggesting an imperfect crystalline structure [39], which did not necessarily affect the RC of the starch samples (Fig. 3-F).

To sum up, considering the results shown in Fig. 3 and the literature [9, 11, 12, 39–41], it is possible to state that the arracacha starches are not amylose-rich, as could be assumed if considered only the apparent amylose analysis. Rather, they have amylopectin with long chain lengths, with the ability to complex the iodine. Also, there are intermediate-sized molecules that can indicate a defective crystalline structure.

In fact, this assumption is corroborated by other results in the present work: the gelatinization temperatures are low (DSC analysis, Table 1), the retrogradation tendency is low (DSC analysis, Table 1, and RVA analysis, Table 2), there was no syneresis even after 30 days of refrigerated storage (data not shown), the apparent viscosity peaks are high (RVA, Table 2) and, even at low temperatures (60 °C), these starches present a remarkable water absorption capacity (Fig. 4-E). Besides, the gels obtained after the gelatinization are elastic (not brittle, as can be seen in Fig. 4-C and D) and present a high paste clarity (discussed in the item 3.2.5). These characteristics give the obtained starches interesting properties, especially from an industrial perspective, as described below.

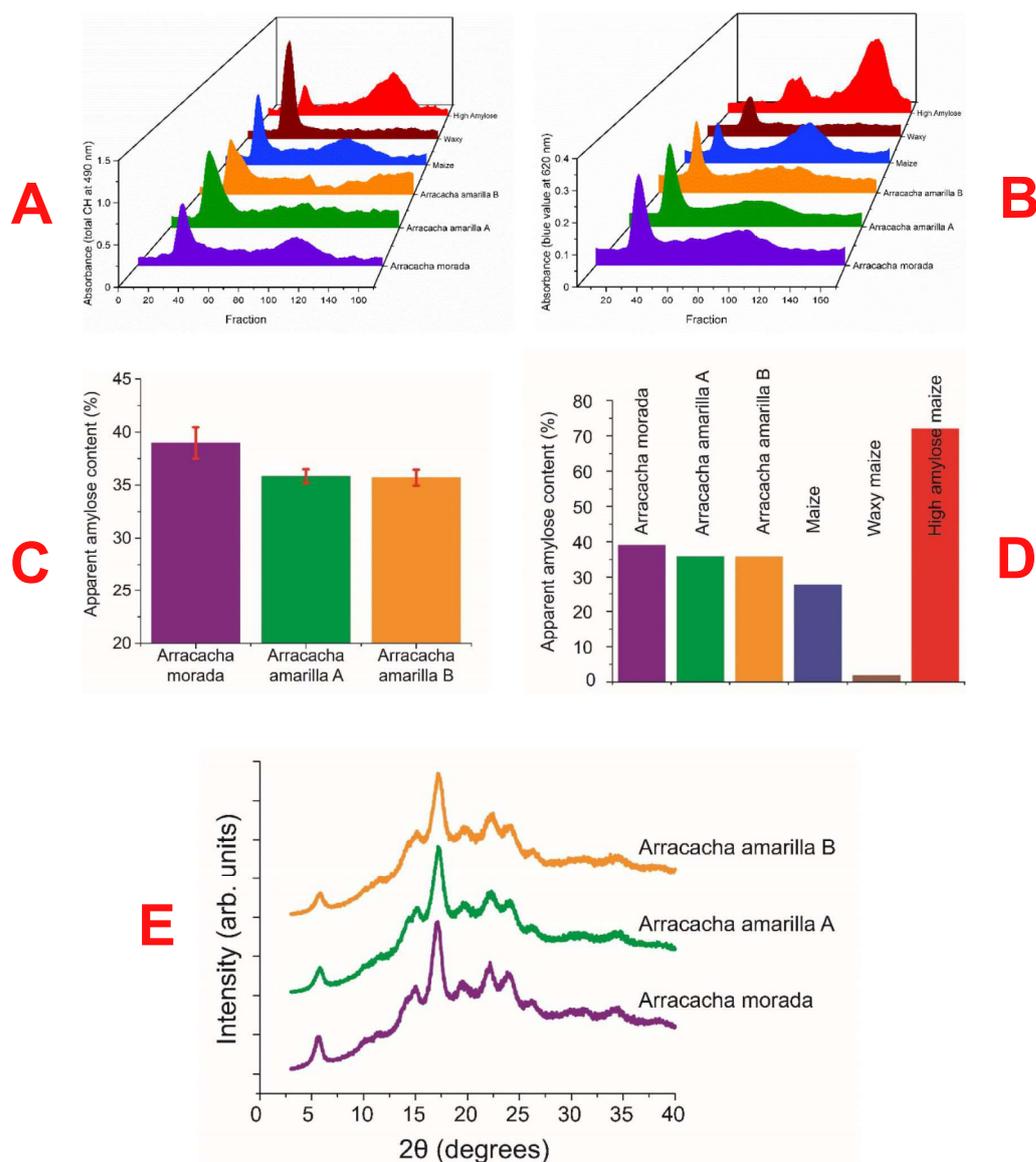


Fig. 3. Molecular size distribution (A, B) and apparent amylose content (C, D) of the arracacha starches and of three varieties of maize starch (normal, waxy and high amylose), and X-ray diffraction patterns and relative crystallinity (E, F) of the arracacha starches. (A) Total carbohydrates chromatogram; (B) Blue value chromatogram; (C) apparent amylose content of arracacha starches; (D) Apparent amylose content comparison among different sources; (E) X-ray diffraction patterns. Red bars represent the standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Gelatinization and retrogradation properties of the arracacha starch samples. Average \pm standard deviations. The percentage retrogradation (%R) was calculated by $[(\Delta H_{\text{retrogradation}}/\Delta H_{\text{gelatinization}}) \cdot 100]$.

Samples	Onset temperature ($^{\circ}\text{C}$)	Peak temperature ($^{\circ}\text{C}$)	Conclusion temperature ($^{\circ}\text{C}$)	Enthalpy (J/g)	%R (%)
Gelatinization					
Morada	53.0 ± 0.6	57.8 ± 0.6	70.4 ± 0.6	6.1 ± 0.9	–
Amarilla A	55.0 ± 0.5	58.9 ± 0.8	71.9 ± 1.8	7.1 ± 0.8	–
Amarilla B	54.9 ± 0.5	59.1 ± 0.4	73.9 ± 0.2	8.8 ± 1.3	–
Retrogradation					
Morada	47.2 ± 0.1	57.5 ± 1.0	66.5 ± 0.2	0.56 ± 0.04	9.2
Amarilla A	48.4 ± 0.5	59.1 ± 0.1	67.5 ± 0.1	0.66 ± 0.01	9.3
Amarilla B	51.8 ± 0.0	59.5 ± 0.2	69.2 ± 2.4	0.56 ± 0.05	6.3

Table 2RVA parameters of the arracacha starch samples. AV = Apparent Viscosity. Average \pm standard deviations.

Test conditions	Sample	Peak AV (mPa·s)	Trough AV (mPa·s)	Breakdown (mPa·s)	Final AV (mPa·s)	Setback (mPa·s)	Pasting temperature (°C)
30-95-30 °C	Arracacha morada	10,708 \pm 95	2135 \pm 12	8573 \pm 83	5155 \pm 147	3021 \pm 159	57.6 \pm 0.8
	Arracacha amarilla A	10,833 \pm 33	1942 \pm 45	8891 \pm 11	4631 \pm 12	2689 \pm 57	58.8 \pm 0.0
	Arracacha amarilla B	10,429 \pm 221	1898 \pm 91	8532 \pm 129	4344 \pm 57	2446 \pm 148	59.1 \pm 0.4
50-95-50 °C	Arracacha morada	10,684 \pm 136	2016 \pm 6	8668 \pm 130	3346 \pm 99	1331 \pm 93	56.5 \pm 0.3
	Arracacha amarilla A	10,720 \pm 74	1851 \pm 18	8869 \pm 92	3015 \pm 21	1164 \pm 2	57.5 \pm 0.1
	Arracacha amarilla B	10,989 \pm 84	1789 \pm 62	9200 \pm 23	2904 \pm 29	1115 \pm 33	58.4 \pm 0.0

3.2. Arracacha starches structure-properties relationship

The properties of the starch are closely related to the structure of the starch molecules. The properties of the arracacha starches are described in the next item and illustrated in Fig. 4. Furthermore, whenever possible, they are related to the structure of the starches described in the previous section.

3.2.1. Thermal properties

The gelatinization and retrogradation properties of the arracacha starches are shown in the Table 1 and illustrated in Fig. S2 (supplementary material).

Starch gelatinization is a complex phenomenon that occurs in the presence of sufficient amounts of heat and water, following some steps: the intermolecular bonds of the starch molecules are weakened

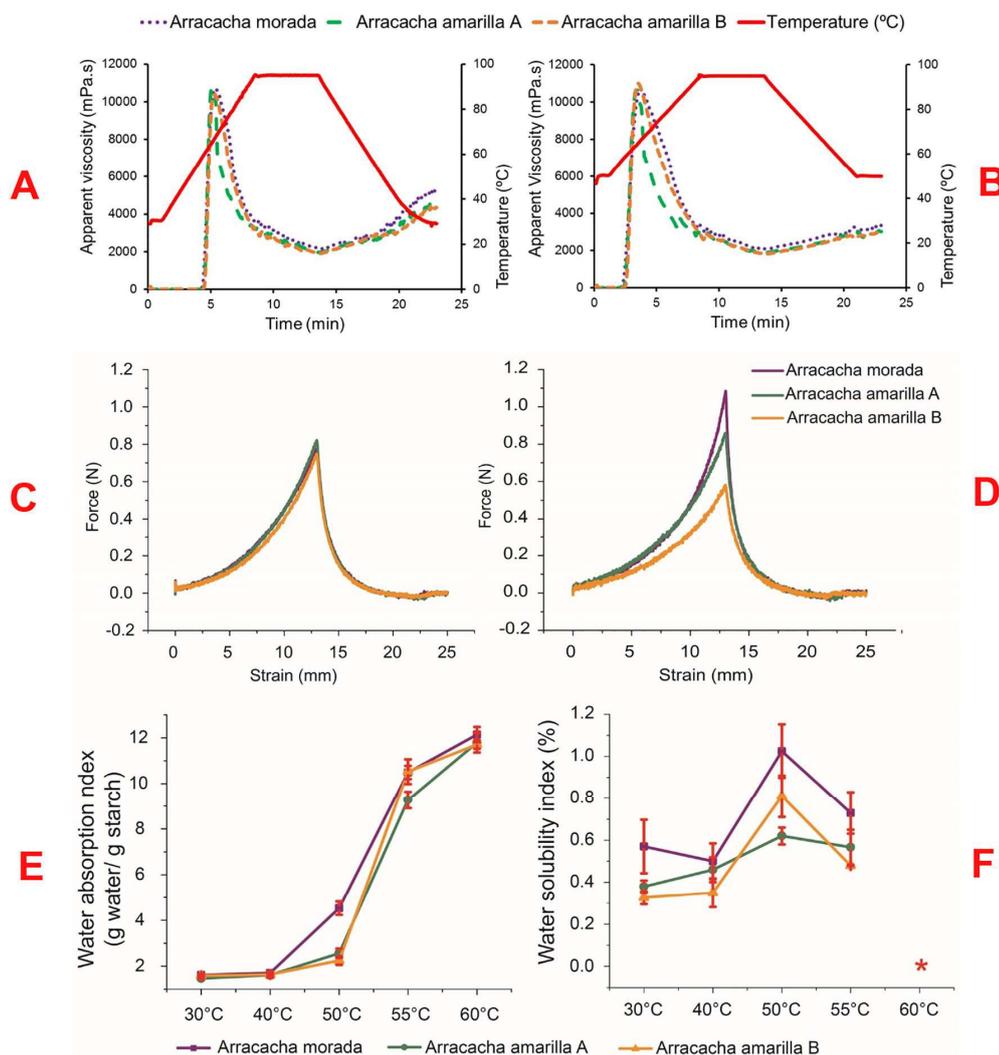


Fig. 4. RVA curves of the arracacha starch samples (A, B), strength of the arracacha starch gel samples (C, D) and Water absorption index and water solubility index of the arracacha starches in different temperatures (30, 40, 50, 55 and 60 °C) (E, F). (A) RVA at the heating program: 30 °C - 95 °C - 30 °C; (B) RVA at the heating program: 50 °C - 95 °C - 50 °C; (C) Gel obtained after the RVA heating cycle illustrated in A; (D) Gel obtained after the RVA heating cycle illustrated in B; (E) Water absorption index; (F) water solubility index. The red * symbol indicates no detectable value. Vertical red bars represent the standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and water penetrates the starch granules, being able to bond with the molecular hydroxyl groups. In this process, the starch granules swell and lose their birefringence, becoming more and more fragile until they disintegrate [43]. Therefore, if the starch structure is naturally less compact (more “fragile”), less energy (heat) is necessary to cause a bond weakening.

In fact, the arracacha starches present a low onset temperature (approx. 53 to 55 °C) and a low enthalpy of gelatinization (approx. 6 to 9 J/g) if compared to other starch samples, such as potato (58.2 °C; 15.8 J/g), normal maize (64.1 °C; 12.3 J/g), waxy maize (64.2 °C; 15.4 J/g) and cassava (64.3 °C; 14.7 J/g) starches [39]. This result is in accordance with the imperfect crystalline structure of their granules (as discussed in the 3.1.3). Besides, the arracacha starch granules present cracks and fissures on their surfaces (Fig. 2), which may facilitate the uptake of water in their structure and, consequently, their gelatinization at lower temperatures.

The observed percentage retrogradation values were small, being close to those observed by Jane et al. [39] for waxy rice (5%) and waxy amaranth (5.2%) starches. Other starch samples showed higher retrogradation values, including potato (43.4%), tapioca (25.3%) or even waxy maize (61.6%) [39]. It clearly indicates that not only the amylose/amylopectin proportion, but also their chain lengths, may influence the retrogradation properties of the starch samples. In the case of the arracacha starches, the large amount of long-branched chains of amylopectin, which also have a heterogeneous distribution of size, may hinder to some extent the molecular reassociation after cooling, contributing to the low retrogradation tendency of the arracacha starches. Besides, to a degree, the phosphate groups may influence the low retrogradation tendency, since electronegative groups also hinder molecular reassociation [28].

Summarizing, when compared with common industrial sources, the arracacha starch thermal properties are characterized by low gelatinization temperatures, gelatinization with a small amount of energy and low retrogradation and syneresis. Consequently, the arracacha starches show a behavior of high industrial interest (especially for applications where refrigeration is used).

3.2.2. Pasting properties

The pasting properties of the arracacha starch samples are given in Table 2 (AV = apparent viscosity), and their relative curves are represented in Fig. 4-A and B.

In the RVA analysis, a sequence of events occurs. First, the starch granules swell until their maximum capacity (reaching the peak AV), followed by a leaching of polymers from the granules and a granular disruption. The leached polymers then suffer an alignment with consequent apparent decrease in viscosity (reaching the trough AV) and, after cooling, there is a reassociation of the molecules with an increase in the apparent viscosity of the system (reaching the final AV). The difference between the peak and trough AVs is known as breakdown and indicates, in a simplified way, the resistance of the starch granules to disruption. The difference between the final and trough AVs is known as setback, and indicates the tendency for re-association of the starch amylose molecules [40].

The RVA analysis was performed in two different heating programs, starting and ending at 50 °C (standard analysis) and at 30 °C. The latter temperature was selected because the gelatinization temperatures of the arracacha starches range between 55 and 60 °C (Table 2). Therefore, a cooling temperature close to 50 °C could be too high to evaluate the possible retrogradation tendency (setback) of these starches. In any case, the setback values of the starches were lower than those observed in samples with a high retrogradation tendency, such as maize and rice starches [44]. In fact, the low retrogradation tendency of the arracacha starches was also observed in the DSC analysis.

Two other characteristics that can be emphasized in relation to the pasting properties of the arracacha starches are their low gelatinization temperature (as discussed in the DSC analysis) and their high Peak AV

(with values ranging between 10,500 and 11,000 mPa·s, as shown in Table 2). The Peak AV values are almost as expressive as those observed in potato starches (~12,500 mPa·s, Castanha et al. [16]), a source that is known for its high Peak AV. This expressive Peak AV is heavily influenced by the amylopectin content of the granules, which contributes to granule swelling [45]. Further, the phosphorus content of the arracacha starches, although not as expressive as in potato starch (as previously discussed), may also influence this result.

To sum up, considering the arracacha starch pasting properties, besides the low gelatinization temperatures and low retrogradation and syneresis (discussed in the DSC analysis), they presented a high Peak AV, which can be useful in applications where a highly viscous paste is interesting – for example, instant soups.

3.2.3. Water absorption and solubility indexes

The water absorption (WAI) and water solubility (WSI) indexes of the arracacha starches are presented in Fig. 4-E and F.

At room temperature, the WAI of the starches is related to the amorphous regions of the granules, since the crystalline regions are too compact to permit the entry of water [40], this condition being altered at the beginning of the gelatinization process.

The WAI is an indirect measure of the swelling capacity of the starch granules. At 30 and 40 °C, the WAI in the granules was negligible, indicating that, at these temperatures (below the onset temperatures measured in the DSC analysis), the starch granules were not able to absorb and/or retain any water. At 50 °C, which is slightly higher than the onset temperature of the samples, it was possible to observe an increase in the WAI, especially for the arracacha morada starch sample (which also had the lowest onset temperature). After this “changing-point”, the WAI of the starches grew sharply, reaching its peak at 60 °C under the conditions studied.

Considering the solubility results, at 30 and 40 °C the soluble portion of the samples did not present large variations. The WSI presented a slight increase with increasing temperature (50 °C), which can be explained by the amylose fractions that may have leached from the granules at this temperature. However, as discussed earlier, as the temperature increased, the WAI of the starch samples also increased. Consequently, at 55 °C there was less available water in the system and, therefore, there was a lower soluble portion at this temperature if compared to 50 °C, explaining the lower WSI. Similarly, at 60 °C all water available for gelatinization was completely absorbed by the granules, and there was no soluble part to evaluate. For this reason, under these analysis conditions (0.5 g of starch in 6 mL of water), the samples were evaluated until 60 °C.

For comparison, we evaluated potato starch under the same conditions: the water absorption capacity of potato starch was below 4 g water/g starch until 60 °C, reaching a value of 8.4 g water/g starch at 95 °C. Consequently, the potato starch water absorption capacity at 95 °C is significantly below the capacity of the arracacha starch even at 60 °C (approx. 12 g water/g starch, or 100% of the available water), highlighting this property in arracacha starch.

To resume, the arracacha starches presented a high water absorption capacity even at low temperatures (60 °C) which, combined with their low retrogradation tendency, may confer desirable characteristics for industrial applications. For example, we consider products where a high incorporation of water is desirable, without affecting texture – which is the case of many commercial food products, such as meat products.

3.2.4. Gel texture properties

The gel strength of the arracacha starch samples is shown in Fig. 4-C and D.

The gels obtained after the RVA analysis were stored for 24 h at 5 ± 2 °C for reassociation of the starch molecules (especially amylose) and consequent stabilization of the gel. After that, a puncture assay was used to evaluate their texture. In general, the gels of all the samples

were smooth and elastic (not brittle). It is possible to observe that there was no difference in the gel strength of the arracacha starch samples obtained in the first heating cycle (30–95 °C). On the other hand, the gel from the arracacha morada sample was slight harder than the others in the standard heating cycle (50–95 °C).

Considering the other analysis, it is possible to observe that the apparent amylose content of the arracacha morada is higher than the arracacha amarilla (Fig. 3-C), and at 50 °C, the morada sample presented a higher soluble fraction (Fig. 4-F). As discussed, the apparent amylose content must be interpreted with caution. However, the analysis may still be used as an indicator for the order of magnitude of the amylose values among the arracacha starch samples. Considering all the information, arracacha morada may have a higher amylose fraction, which leached from the granule at 50 °C (but not at 30 °C) and, therefore, presents a higher reassociation after cooling, forming a more rigid gel if compared to the other arracacha starch samples.

However, if compared to other starch samples analysed under the same conditions, we observe that the gels from arracacha starches are softer than those from maize starch, (which required more than 3 N to be fully penetrated), or potato starch (demanded almost 2 N), but harder than those from cassava starch (requiring 0.4 N).

In short, for products where a non-brittle, soft and minimally consistent (with shaping capacity) gel is required, arracacha starch may be a good choice. Examples can be many refrigerated desserts, such as puddings (especially considering the other cited properties).

3.2.5. Paste clarity

The clarity of the arracacha starch pastes was over 90% of transmittance (100% being the equivalent of the transmittance of distilled water) for all samples: 96.1 ± 0.1% for arracacha morada, 92.8 ± 2.8% for the arracacha armilla A and 93.4 ± 2.9% for the arracacha amarilla B.

These results indicate a high paste clarity. In fact, the clarity of the paste from arracacha starches is comparable to potato starch paste (approx. 94%, Castanha et al. [16]), one of the clearest pastes for a native starch.

This high paste clarity can be attributed to different factors, including the high swelling capacity (WAI, Fig. 4-E), repulsion between negatively charged molecules (phosphate groups, Table S1) and low retrogradation tendency (DSC, Table 1 and RVA, Table 2) [28].

Considering the applications of starch, these are promising results, especially for the food, paper and textile industries, where a high paste clarity may be highly desirable. Furthermore, a clear paste obtained through a native (non-modified) starch can also increase the “natural” claim for the products where it is applied.

4. Conclusion

Considering the starch characteristics, both arracacha varieties (morada and amarilla) have an intermediate phosphorus content, and granules with an average size of about 20 µm, round-shaped (with smaller granules with polygonal shapes) and with some cracks and fissures on their surface. Also, the granules present a B-type pattern (typical of tubers and roots).

The apparent amylose content (based on iodine affinity) observed in the arracacha starch sample was high. However, the size-exclusion chromatography demonstrated amylopectin molecules with long chains with the ability to complex the iodine. Also, intermediate-sized molecules of amylopectin were observed, indicating a defective crystalline structure.

Considering the properties of the arracacha starches, they presented a low gelatinization temperature, a low enthalpy of gelatinization, a low retrogradation tendency and a high Peak apparent viscosity. These results were described based on the starch structure, and they are very interesting from an industrial perspective.

The water absorption and water solubility indexes indicated a high swelling capacity of the arracacha starches even at moderate

temperatures (60 °C). Besides, the arracacha starch gels presented a smooth and “elastic” (not brittle) texture and a high paste clarity.

By comparing the characteristics of the two arracacha varieties studied in this paper, as well as by comparing the two starch samples from the “amarilla” variety, no expressive differences were observed among them.

Summarizing, the most relevant aspects of the arracacha starches were their low gelatinization temperature, low retrogradation tendency, high water absorption capacity and high paste clarity. From the industrial point of view, all these aspects being present in a non-modified starch may be desirable for many applications, especially for the food industry, since the native starches give a “natural” claim to processed foods.

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APPENDIX B: Effect of concentration on the pasting properties and gel strength of starches from different sources

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28 the best conditions for different industrial applications, as well as to demonstrate how
29 small differences in the pasting parameters can influence the starch properties.

30

31 **Keywords:** properties; gelatinization; texture; application; evaluation conditions

32

33 **Practical Applications:**

34 In this work, starches from five different sources were evaluated under non-standard
35 conditions of analyses, in order to simulate different industrial applications. The most
36 important parameters to evaluate the quality of starchy products were considered. We
37 demonstrated that decisions of industrial application based on the results of standard
38 protocols may not suit the real necessities, leading to misinterpretations. The results
39 reported in this work are useful for the selection of the best conditions of evaluation and
40 also application for different academic and industrial applications.

41

42 **1. Introduction**

43 Starches are a versatile and widely used material, with undeniable economic
44 importance. They are composed mainly by two polysaccharides, amylose and
45 amylopectin, that are organized in complex structures called granules. Different sources
46 present different molecular and granular characteristics; consequently, different
47 properties are available depending on the starch source and process conditions. The
48 main characteristics of the starch structure are well established in the literature, and can
49 be found elsewhere [1–5].

50 Starch gelatinization is one of the most explored phenomena in starch science,
51 and has been explored in both classical [6–10] and current literature [11–13].

52 However, despite the massive amount of works on this subject, and despite the
53 different techniques employed aiming to better characterize the gelatinization behaviour
54 of different starch sources, this phenomenon can still be unpredictable in different
55 conditions. An evidence of this unpredictability is the raising of recent articles whose
56 purpose is to model the gelatinization stages of different starches [14,15], which proves
57 that it is a still unsolved problem.

58 Meantime, the importance to understand the gelatinization process and how it
59 affects the starch properties goes beyond the scientific approach. The functional
60 properties of different starchy food products, as their texture and digestibility, are closely
61 related to the gelatinization and retrogradation processes. Consequently, most of the
62 industrial applications involve gelatinized starches, or their gelatinization.

63 It is important to highlight that, although there are several ways to evaluate the
64 rheological behaviour of starchy dispersions and solutions, their evaluation using a Rapid
65 Visco Analyser (RVA) is one of the most popular. Furthermore, the majority of this assays
66 are performed using the RVA standard protocols of analysis, such as standard
67 concentration (usually fixed at 10.7% m/m, corrected to 14% moisture basis) and
68 temperature.

69 It is comprehensible that it is necessary to standardize the analysis conditions in
70 order to ensure a fair comparison between different samples. This occurs since the
71 behaviour of each starch source under different process conditions is difficult to precisely
72 determine, since it is influenced by several factors, as temperature rate, starch
73 concentration and shear forces [15]. Furthermore, different starch sources imply in
74 different particles size, granules morphology, molecular weight and amylose/amylopectin
75 ratios, among others, which completely changes the gelatinization behaviour of the
76 samples [7].

77 However, the standard protocols of analysis may not be representative for all
78 industrial applications, and evaluation at different conditions may be important to
79 understand the starch behaviour and to propose possible applications.

80 In this work, starches from different sources were evaluated regarding their
81 gelatinization properties. For this purpose, the pasting properties (using an RVA
82 equipment) and the gel strength (using a texture analyser) of the starch samples were
83 evaluated under different starch concentrations and compared to the typical analysis
84 conditions, aiming to provide new data for the characterization of these starches.

85

86 **2. Material and methods**

87

88 **2.1. Material**

89 The potato (*Solanum tuberosum* L., Monalisa cultivar) starch sample (presenting
90 ~28% of apparent amylose) was extracted and characterized according to the described
91 in Castanha et al. [16]. The maize starch (~28% of amylose, Argo CS 3400), high-
92 amylose maize starch (~72% of amylose, Hylon VII), waxy maize starch (~98% of
93 amylopectin, Amisol 4000) and cassava starch (~20% of amylose) were kindly provided
94 by “Ingredion Brasil Ingredientes Ltda” (Brazil). All the starch samples presented a purity
95 higher than 99%.

96

97 **2.2. Starch evaluation**

98

99 2.2.1. Granules morphology: light microscopy and Particle Size Distribution (PSD)

100 Images of the starch granules were obtained through a light microscope (model
101 L1000 with a 20 W halogen lamp, Bioval, Curitiba, Brazil). The samples were prepared
102 as described by Castanha et al. [16]. The magnification of 400x was used and a portable
103 camera of 1.3 megapixels was used to register representative images.

104 The Particle Size Distribution (PSD) of the starch granules was determined using
105 a Laser Analyser (Partica LA-950V2 Laser Particle Size Analyser HORIBA, Japan),
106 according to the described by Castanha et al. [17], using ethanol (99.5%) to disperse the
107 samples. The software LA-950 for Windows (HORIBA, Japan) was used to calculate the
108 distribution of the particles, obtained through the volume-based mean diameter.

109

110 2.2.2. Pasting properties

111 The starch pasting properties were determined using a Rapid Visco Analyser
112 (RVA, model RVA-4, Newport Scientific Pvt. Ltd., Australia, with the Thermocline for
113 Windows software version 3.0). Different starch/distilled water proportions were used: 1
114 g /27 g (3.6%), 2 g /26 g (7.1%), 3 g /25 g (10.7%, being this concentration used in the
115 standard protocols of the RVA equipment) and 4 g /24 g (14.3%). The starch mass was
116 always corrected to 14% moisture basis.

117 Further, two different temperature combinations were used in this work, aiming
118 to evaluate the effect of the initial and final temperatures on the starch gelatinization
119 process. In the first combination, the suspensions were first held at 50°C for 1 min, then
120 heated until 95°C during 7 min and 30 s, being then kept at this temperature for 5 min,
121 followed by cooling to 50°C for 7 min and 30 s, and finally hold at 50°C for 2 min. This
122 first combination is the standard protocol of the RVA equipment. In the second

123 combination, the initial and final temperatures of the RVA protocol were lowered from
124 50°C to 40°C.

125

126 2.2.3. Gel strength

127 The starch gel strength was evaluated through a puncture assay using a Texture
128 Analyser (TA.XT Plus, Stable Micro Systems Ltd., Surrey, UK; load cell of 50 kgf /490.3
129 N). The gels obtained after the RVA assays were transferred to plastic cups (40 x 20
130 mm, diameter x height) and stored at $5 \pm 2^\circ\text{C}$ for 24 h inside a desiccator with water to
131 ensure uniform moisture distribution. The penetration assay was performed using a 0.5
132 cm cylindrical probe (P/0.5R) at $1 \text{ mm}\cdot\text{s}^{-1}$ until the distance of 15 mm. The force
133 measured by the equipment as a function of the penetration depth was plotted, and the
134 samples strength was evaluated through the total energy required for the penetration
135 (which was obtained through the area below the “force versus penetration” curve).

136

137 2.3. Statistical analysis

138 Each analysis was performed at least in duplicate for each starch sample. When
139 relevant, a statistical analysis was performed on the treatments through analysis of
140 variance (ANOVA) and Tukey’s test, using the Origin software, version 9.1 (Microcal Inc.,
141 Northampton, MA, USA). A significance level of 5% was considered.

142

143 3. Results and discussion

144

145 3.1. Pasting properties

146 The morphology and size distribution (PSD) of the granules of the different starch
147 samples, as well as their pasting properties at the 50-95-50°C assay, are illustrated in
148 Figure 1. Table 1 shows the main parameters obtained from the pasting properties using
149 both temperature protocols, and the pasting properties graphics of all starch samples
150 under all conditions are presented in Figure 2.

151 By analysing the effect of the initial and final temperatures (40 versus 50°C) used
152 in the RVA assays, i.e. comparing the same starch source at the same concentration,
153 most of the samples did not present a significant variation ($p < 0.05$) in their pasting
154 parameters (Table 1). Therefore, the temperature effect will not be deeply explored in
155 this work. On the other hand, the RVA profiles (Figures 1, 2 and Table 1) were distinct
156 and characteristic for each starch source and concentration.

157 In general, the majority of the obtained results presented an expected pattern, as
158 increasing apparent viscosity (AV) with increasing starch concentration. Furthermore, at
159 the standard protocol (10.7% of starch), the analysed samples presented a familiar
160 pasting profile (Figures 1 and 2, Table 1), being the Peak AV per sample ordered as:
161 potato > cassava > waxy maize > normal maize > high amylose maize. This behaviour
162 was highly influenced by the starches intrinsic characteristics, as their granules size and
163 morphology, as well as the amylose/ amylopectin ratio and their respective chain lengths
164 [7,9].

165 At this point, it is important to highlight some of those intrinsic characteristics of
166 the starch here evaluated, which will be useful to understand the observed results. The
167 peaks of the PSD (Figure 1) of the samples, calculated based on the volume-based
168 mean diameter ($D[3,4]$) [18,19], can be ordered as follows: High amylose maize
169 (diameter = 15.2 μm) < normal maize (17.4 μm) < cassava (22.8 μm) < waxy maize (26.1
170 μm) < potato (39.2 μm). The apparent amylose content can be ordered as follows: waxy
171 maize (~2%) < cassava (~20%) < normal maize (~28%) = potato (~28%) < high amylose
172 maize (~72%). Also, the potato starch phosphorus content (~800 mg/kg, Castanha et al.
173 [16]), is much higher than the other starch sources.

174 As can be observed, no direct correlation can be done between the values of
175 peak apparent viscosity and the apparent amylose content or PSD values of the
176 samples. In fact, the swelling behaviour of each starch granule is a complex relation of
177 the amylose/ amylopectin balance and their sizes, as well described and discussed in
178 the literature [7,9,20].

179 The peak AV pattern, however, was not maintained when the starches' proportion
180 were 14.3%, demonstrating that the processing conditions would also be an important
181 factor affecting the starches pasting behaviour (and not only their structure). In fact, even
182 among the samples with "predictable" behaviour, as the ones presenting increasing AV
183 with increasing starch concentration, the increase was not linear or equivalent for the
184 starch samples.

185 By evaluating each starch source separately, we can observe some interesting
186 patterns.

187 Potato starch, for example, presented a "non-standard" shape in its pasting curve
188 at the lower starch concentration (3.6%), without the characteristic "Setback" and
189 "Breakdown" regions. This behaviour occurred only with the potato starch, and thus it
190 cannot be explained only by the lower interactions among the starch granules, which is
191 typical at low concentrations [21,22]. This demonstrates that the interactions of the
192 leached molecules during cooling were expressive, even considering the negligible
193 granules' swelling during pasting. It is interesting to observe that this concentration
194 (3.6%) is closer to the typical used in the industry, such as for mayonnaise [23] or salad
195 dressings [24,25] for example, although the standard protocol uses a concentration of
196 10.7%, which is, in general, much higher than the concentration used for most of
197 industrial food products. Therefore, we highlight that decisions of industrial application
198 based on the results of standard protocols may not suit the real necessities, leading to
199 misinterpretations.

200 Another aspect regarding the potato starch was its behaviour at the highest
201 starch/ water proportion (14.3%). At this condition, the sample presented lower Trough
202 AV and Final AV if compared to the 7.1 and 10.7% samples. These results can be related
203 to a possible lower gelatinization degree of the most concentrated sample, due to the
204 lack of available water and/or due to the potato starch bigger granules (explaining why
205 this behaviour was not observed for the other starch sources). The less swelled/
206 gelatinized starch granules probably influenced in a lower extend the AV of the system,

207 if compared to the more gelatinized granules. In fact, this could be proved by optical
208 microscopy (Supplementary material, Figure S1).

209 Regarding the cassava starch, at 3.6 and 7.1% of starch concentration, its Peak
210 AV was lower than its Final AV, condition that is inversed by increasing the starch
211 concentration. To give a possible explanation to this behaviour, we first need to recall
212 that the RVA analysis measures the apparent viscosity of the system during a heating-
213 cooling process. During this process, the starch granules suffers irreversible changes,
214 and not only their leached amylose and amylopectin molecules, but also the remnant
215 granules, their fragments and ghosts are interconnected and form a complex network
216 (“gel”) that changes the system’s characteristics. Keeping that in mind, it is possible to
217 say that the effect of cooling in the network formation of cassava starch is more
218 significant at lower concentrations (3.6 and 7.1%) than at higher (10.7 and 14.3%).

219 Regarding the waxy and normal maize starches, the importance of the starch/
220 water concentration becomes even more evident. For example, the waxy maize is
221 usually mentioned as presenting a higher Peak AV if compared to the normal maize
222 starch. However, this statement can only be considered true at the lower concentrations
223 (3.6, 7.1 and 10.7%), since at 14.3% the normal maize presented a higher Peak AV value
224 if compared to the waxy sample. The amylopectin molecules are usually related with the
225 peak AV observed in the RVA analysis [4], which explains the waxy maize starch higher
226 peak AV if compared to the normal maize starch. However, the amylose/ amylopectin
227 ratio is a complex and important parameter that can influence more the starch
228 gelatinization behaviour than the amylopectin content by itself [20]. This ratio can
229 possibly explain why at 14.3% the normal maize starch presented a higher viscosity if
230 compared to waxy samples. On the other hand, regarding the Final AV, an opposite
231 behaviour was observed, and the waxy starch only showed a higher Final AV than the
232 normal maize starch at 3.6%. This result also can be explained by the amylose/
233 amylopectin ratio of the maize samples, but in this case the amylose content played a

234 more important role (since it is more related with the Final AV than the amylopectin
235 molecules).

236 The high amylose maize starch presented a negligible AV variation after the RVA
237 assays. Only at 14.3% its AV variation was minimally measurable (being comparable to
238 the AV of the cassava and waxy maize samples at 3.6%). This lower AV is due to the
239 incomplete gelatinization of the high AM maize starch under the RVA pasting conditions
240 (gelatinization temperature < 100 °C). As discussed in the literature [9], the amylopectin
241 molecules have a major contribution on the starch swelling, while the amylose inhibit it.
242 In fact, the high amylose starches (> 50% of amylose content) are usually reported to
243 require a high temperature (> 130°C) to be completely gelatinized [7,26–28].

244 In summary, when comparing the AV values of starch, each source presented a
245 different behaviour in relation to the starch proportion variation. Considering the peak
246 AV, for example, the cassava starch showed a decrease of ~70% on the peak AV value
247 when the starch concentration was reduced from 10.7% to 7.1%, and an increase of
248 ~111% when the concentration was increased from 10.7% to 14.3%, while the other
249 starch samples showed very different behaviours. At the same conditions, the maize
250 starch presented a decrease of ~71% and an increase of ~141%, the waxy maize starch
251 a decrease of ~58% and an increase of ~54%, and the potato starch a decrease of ~65%
252 and an increase of ~29%. All those results illustrate once again that the 10.7% of starch
253 concentration is not always predictive for the starch behavior in all possible applications.

254

255 **3.2. Gel strength**

256 Figure 3 illustrates the gel strength of the samples obtained after each RVA
257 assay. Since no significative differences were observed when comparing the samples
258 obtained though the different RVA temperature protocols (using 40 and 50°C), only the
259 results referent to the gels obtained using the RVA protocol at 50°C are presented in
260 Figure 3.

261 As in the pasting properties results, the general (and expected) behaviour of the
262 samples was to present an increase in the gel strength with increasing starch
263 concentration. Some aspects, however, worth be highlighted.

264 The high amylose maize starch presented a weak gel, due to its incomplete
265 gelatinization, as previously discussed for the RVA analysis. On the other hand, the waxy
266 maize starch, despite completely gelatinized, also presented an extremely weak gel,
267 exhibiting a negligible strength (with its highest value of ~0.6 mJ). Considering that the
268 gel formation depends on a complex network between amylose and amylopectin
269 molecules that leached the granules during the gelatinization, as well as the remnant
270 granules and their fragments, it is possible to say that the weak texture presented by the
271 waxy starch was due to the lack of amylose molecules in this sample. In fact, the amylose
272 molecules are highly correlated to the gel formation after cooling [30,31].

273 Similarly, the cassava starch presented smooth and elastic gels, with extremely
274 low resistance to the penetration test (highest value of ~2 mJ in Figure 3). In this case,
275 the low amylose content (~20%) can also be related to this weak gel. However, another
276 factor can be involved, as the high amylopectin chain-length, which can impair to some
277 extent the molecular re-association of the cassava sample after cooling.

278 The gel strength of the potato and the maize starches was similar in almost all
279 starch concentrations (except at 3.6%), despite the high differences among their pasting
280 properties. Both presented the highest gel strength values among all samples. This can
281 be attributed to their similar amylose content (~28%), which is mainly related to the short-
282 period gel formation [30,31]. On the other hand, the difference observed in their pasting
283 properties can be attributed to their different granule size and phosphorus content, which
284 is high in the potato starch and is frequently attributed to enhance the apparent viscosity
285 [32].

286 In fact, the pasting profile of the starch samples cannot be used as the only
287 parameter for prediction of the gel formation and/or strength after cooling. In the case of
288 the gel, in special regarding its development after a short storage period, the amylose

289 content and its leaching of the granules plays an important role [30,33]. The cassava
290 starch, for example, presented the second highest peak AV of all samples, in all
291 concentrations. However, its gel presented a negligible strength, illustrating that the
292 pasting profile cannot be used to predict the behaviour of the starch gel after cooling and
293 storage.

294 Summarizing, considering all the results and the literature data, the starch
295 gelatinization is a complex phenomenon that is affected by several intrinsic and extrinsic
296 parameters. Therefore, despite the need of a standard protocol to allow a fair comparison
297 among different starch samples, native or modified, the results obtained through these
298 typical analyses cannot be settled as unique or always true. In fact, the starches
299 behaviour under different processing conditions are very difficult to predict and control,
300 and worth be individually investigated.

301 The exact role that each parameter plays and how much they influence the final
302 characteristics of the starch samples remain unclear. Therefore, further studies, using
303 different analysis conditions, are important in order to better correlate the relationship
304 between the starch structure and properties with the processing conditions.

305

306 **4. Conclusion**

307 In this work, the effect of different concentrations on the pasting properties and
308 gel strength of five different starch sources were studied. It was shown that using only at
309 a typical condition (concentration of 10.7%) may not be appropriate to evaluate the starch
310 behaviour in all possible applications. By varying the starch concentration, not only the
311 apparent viscosity value during RVA varied, but also the behaviour and shape of their
312 pasting curves. Furthermore, the different starch concentrations influenced the strength
313 of the obtained gels.

314 In fact, the conditions in which a starch sample will be applied in a product must
315 be considered as important as the starch source itself. The results reported in this work
316 provides new data for the characterization of different starch samples under non-

317 standard conditions of analysis, expanding the amount of results that are available for
318 future reference. In addition, these results can be useful for the selection of different
319 parameters for the industrial applications of the analysed starches.

320

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334

335 **Conflict of interest statement**

336 The authors have declared no conflict of interest.

337

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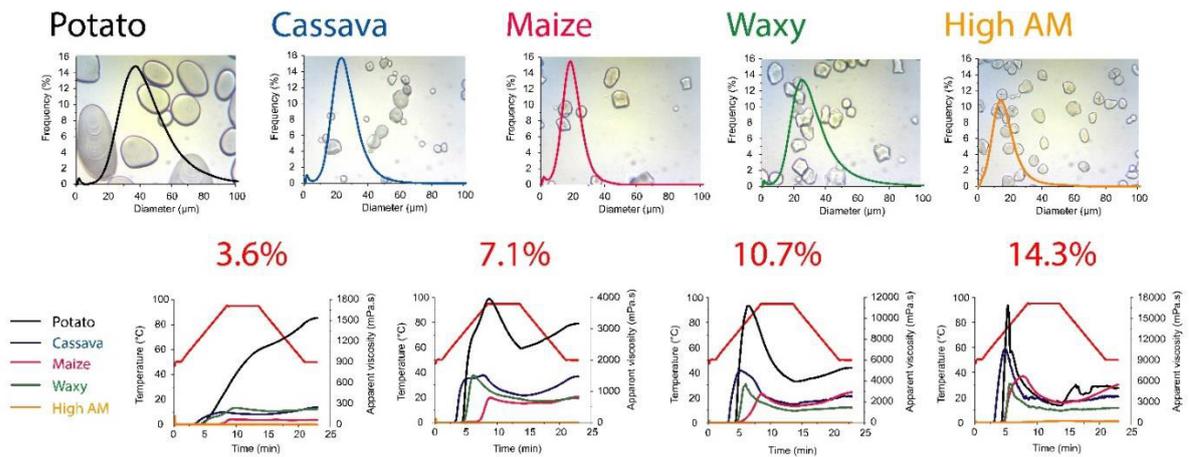


Figure 1: Top line: Granules morphology (microscopy) and particle size distribution (PSD) of the different starch samples. Bottom line: pasting properties (RVA) using the standard temperature protocol (50-95-50°C) and four different starch concentrations (3.6%, 7.1%, 10.7% and 14.3%) of the different starch samples. AM = amylose.

Table 1: Main parameters of the pasting properties of each starch source under different analysis conditions (concentration and temperature). Average \pm standard deviations. AV = apparent viscosity. Samples followed by the same letters do not differ significantly at 95% of confidence (statistical analysis performed between the same starch sources).

Samples	Conditions	Peak AV (mPa.s)	Trough AV (mPa.s)	Breakdown (mPa.s)	Final AV (mPa.s)	Setback (mPa.s)					
Potato	3.6% - 40°C	1095 \pm 53	a	1097 \pm 54	a	-3 \pm 1	a	1746 \pm 42	a	649 \pm 11	ab
	3.6% - 50°C	1040 \pm 50	a	1045 \pm 50	a	-5 \pm 0	a	1539 \pm 21	a	495 \pm 29	a
	7.1% - 40°C	4069 \pm 30	a	2253 \pm 73	b	1817 \pm 43	ab	3601 \pm 18	b	1348 \pm 55	bc
	7.1% - 50°C	3961 \pm 17	a	2373 \pm 40	b	1588 \pm 23	ab	3167 \pm 45	bd	794 \pm 6	ab
	10.7% - 40°C	11319 \pm 73	b	3792 \pm 19	c	7527 \pm 54	bc	5808 \pm 59	c	2016 \pm 78	cd
	10.7% - 50°C	11225 \pm 178	b	3938 \pm 23	c	7287 \pm 156	bc	5256 \pm 27	ce	1318 \pm 4	
	14.3% - 40°C	17323 \pm 765	b	2220 \pm 98	b	15104 \pm 667	c	2596 \pm 586	d	376 \pm 488	ab
14.3% - 50°C	17973 \pm 1181	b	2355 \pm 75	b	15618 \pm 1256	c	4900 \pm 69	e	2545 \pm 6	d	
Cassava	3.6% - 40°C	184 \pm 1	a	148 \pm 1	a	36 \pm 1	a	303 \pm 3	a	155 \pm 1	a
	3.6% - 50°C	182 \pm 16	a	148 \pm 11	a	34 \pm 4	a	251 \pm 13	a	103 \pm 2	a
	7.1% - 40°C	1504 \pm 8	b	866 \pm 9	b	639 \pm 1	b	1951 \pm 38	b	1086 \pm 29	b
	7.1% - 50°C	1514 \pm 21	b	867 \pm 4	b	647 \pm 17	b	1479 \pm 11	c	612 \pm 7	c
	10.7% - 40°C	4996 \pm 51	c	1769 \pm 4	c	3227 \pm 47	c	3085 \pm 85	d	1316 \pm 81	d
	10.7% - 50°C	4986 \pm 16	c	1714 \pm 18	c	3273 \pm 2	c	2522 \pm 118	e	808 \pm 100	ce
	14.3% - 40°C	10650 \pm 151	d	2858 \pm 6	d	7792 \pm 144	d	4601 \pm 42	f	1744 \pm 36	f
14.3% - 50°C	10524 \pm 45	d	2807 \pm 33	d	7717 \pm 11	d	3710 \pm 126	g	904 \pm 93	be	
Maize	3.6% - 40°C	77 \pm 3	a	64 \pm 3	a	13 \pm 0	a	75 \pm 5	a	11 \pm 2	a
	3.6% - 50°C	78 \pm 11	a	65 \pm 8	a	13 \pm 3	a	71 \pm 8	a	6 \pm 0	a
	7.1% - 40°C	796 \pm 6	b	615 \pm 8	b	181 \pm 3	b	929 \pm 20	b	314 \pm 11	a
	7.1% - 50°C	797 \pm 3	b	615 \pm 1	b	182 \pm 1	b	822 \pm 7	b	207 \pm 6	a
	10.7% - 40°C	2695 \pm 23	c	1559 \pm 43	c	1136 \pm 66	c	3523 \pm 1	c	1965 \pm 42	b
	10.7% - 50°C	2744 \pm 4	c	1565 \pm 54	c	1179 \pm 58	c	2913 \pm 8	c	1348 \pm 46	c
	14.3% - 40°C	6660 \pm 11	d	2842 \pm 2	d	3818 \pm 13	d	6797 \pm 370	d	3955 \pm 368	d
14.3% - 50°C	6627 \pm 16	d	2780 \pm 33	d	3848 \pm 49	d	5430 \pm 23	e	2651 \pm 11	e	
Waxy	3.6% - 40°C	249 \pm 5	a	196 \pm 4	a	53 \pm 1	a	249 \pm 10	a	53 \pm 6	a
	3.6% - 50°C	243 \pm 0	a	194 \pm 1	a	49 \pm 1	a	227 \pm 4	a	33 \pm 2	a
	7.1% - 40°C	1513 \pm 12	b	674 \pm 1	ab	839 \pm 13	b	854 \pm 1	b	180 \pm 1	a
	7.1% - 50°C	1508 \pm 17	b	678 \pm 5	ab	831 \pm 22	b	772 \pm 6	b	95 \pm 11	a
	10.7% - 40°C	3447 \pm 62	c	1067 \pm 3	c	2380 \pm 59	c	1724 \pm 22	d	541 \pm 19	ab
	10.7% - 50°C	3593 \pm 147	c	1122 \pm 17	bc	2471 \pm 164	c	1424 \pm 44	c	302 \pm 61	ab
	14.3% - 40°C	5693 \pm 37	d	1657 \pm 23	c	4036 \pm 59	d	2385 \pm 45	e	728 \pm 23	b
14.3% - 50°C	5588 \pm 21	d	1655 \pm 13	c	3933 \pm 34	d	2078 \pm 10	f	424 \pm 4	ab	
High amylose	3.6% - 40°C	3 \pm 1	a	-1 \pm 1	a	3 \pm 1	a	2 \pm 1	a	3 \pm 1	a
	3.6% - 50°C	6 \pm 8	ac	2 \pm 6	a	4 \pm 2	a	3 \pm 5	a	1 \pm 1	a
	7.1% - 40°C	7 \pm 1	ac	6 \pm 1	a	2 \pm 1	a	8 \pm 1	a	2 \pm 0	a
	7.1% - 50°C	6 \pm 4	ac	5 \pm 4	a	2 \pm 1	a	6 \pm 2	a	1 \pm 1	a
	10.7% - 40°C	31 \pm 4	bc	27 \pm 2	b	4 \pm 1	a	36 \pm 2	b	9 \pm 0	a
	10.7% - 50°C	24 \pm 2	c	21 \pm 2	b	3 \pm 0	a	23 \pm 1	ab	3 \pm 1	a
	14.3% - 40°C	209 \pm 10	d	151 \pm 7	c	58 \pm 3	b	278 \pm 15	c	127 \pm 8	b
14.3% - 50°C	185 \pm 0	e	136 \pm 1	d	50 \pm 1	c	157 \pm 1	d	22 \pm 1	c	

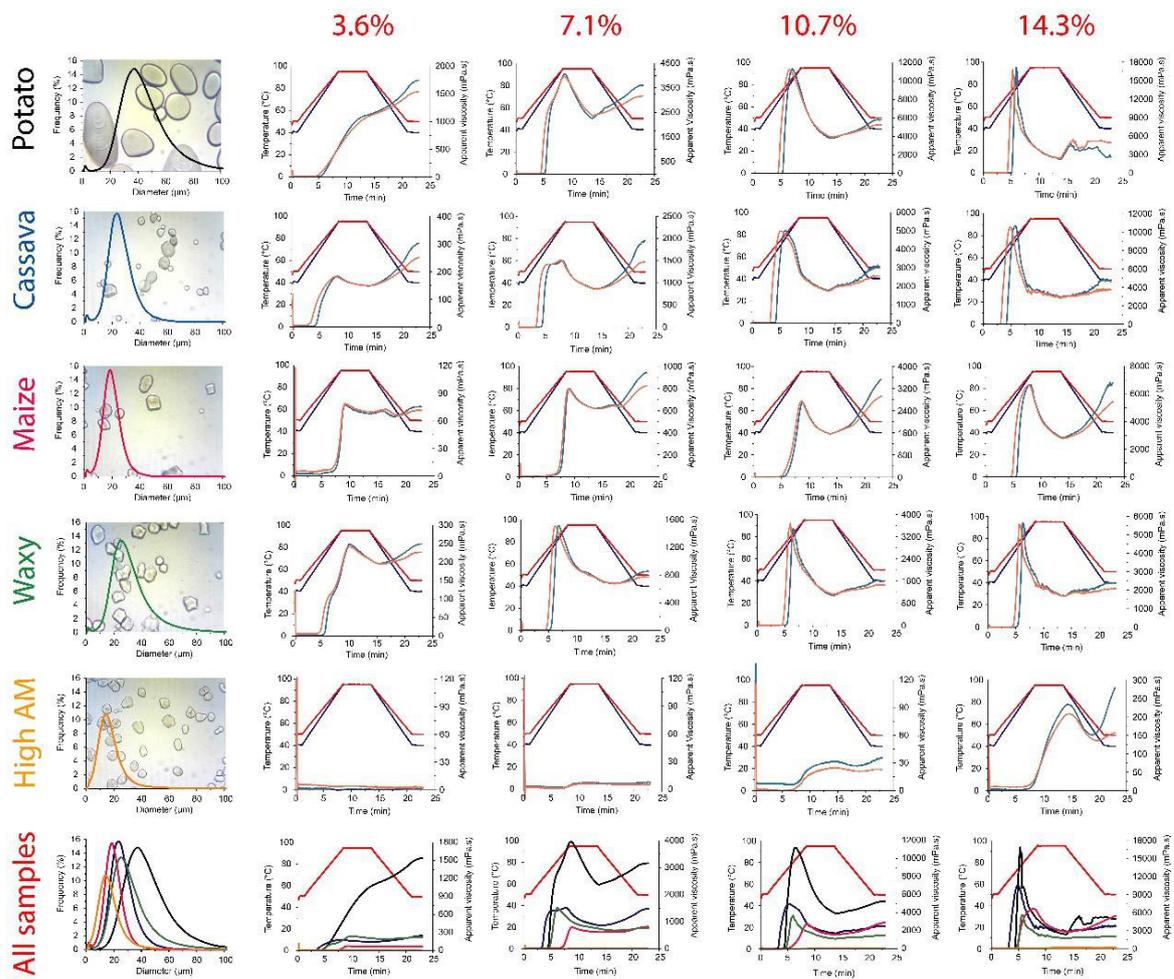


Figure 2: Pasting properties of the different starch samples. From top to bottom: potato, cassava, maize, waxy maize and high amylose maize starches. AM = amylose. The last line illustrates all samples together, being the RVA curves at 50°C. From left to right: RVA curves at 3.6%, 7.1%, 10.7% and 14.3% of starch, considering the assays starting and finishing at 40°C (blue lines) and 50°C (red lines).

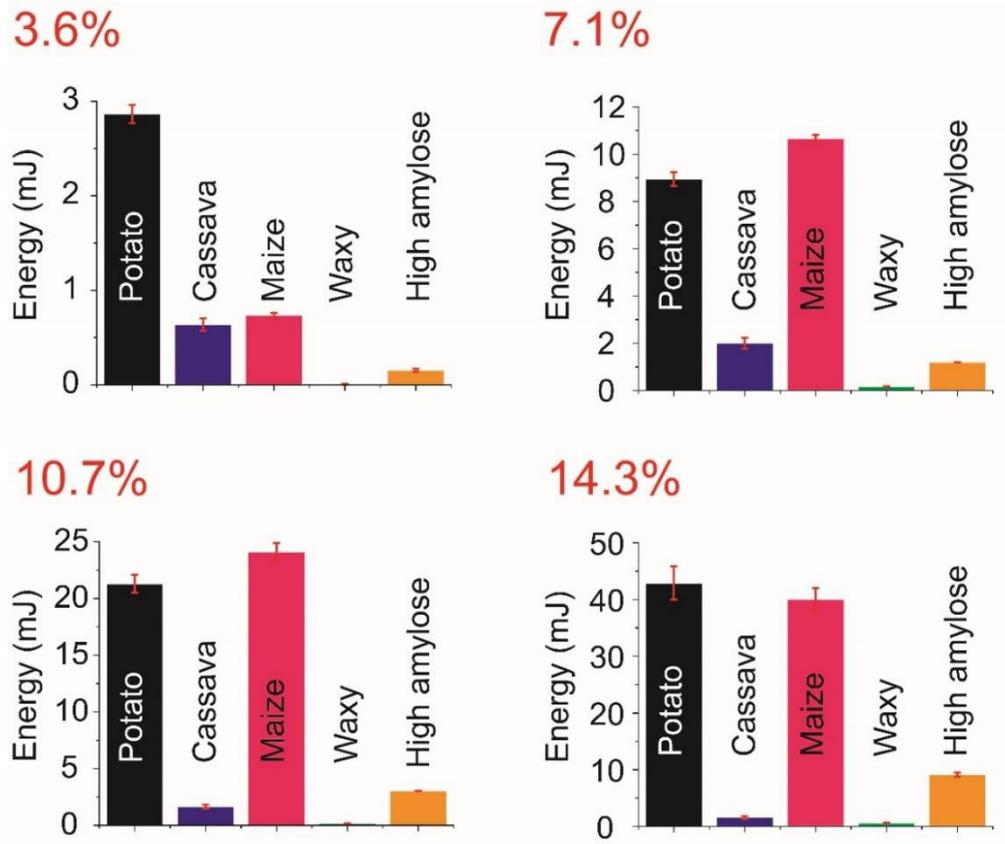


Figure 3: Gel strength of the starch gels obtained after the after the RVA assays using the standard temperature protocol (50-95-50°C).

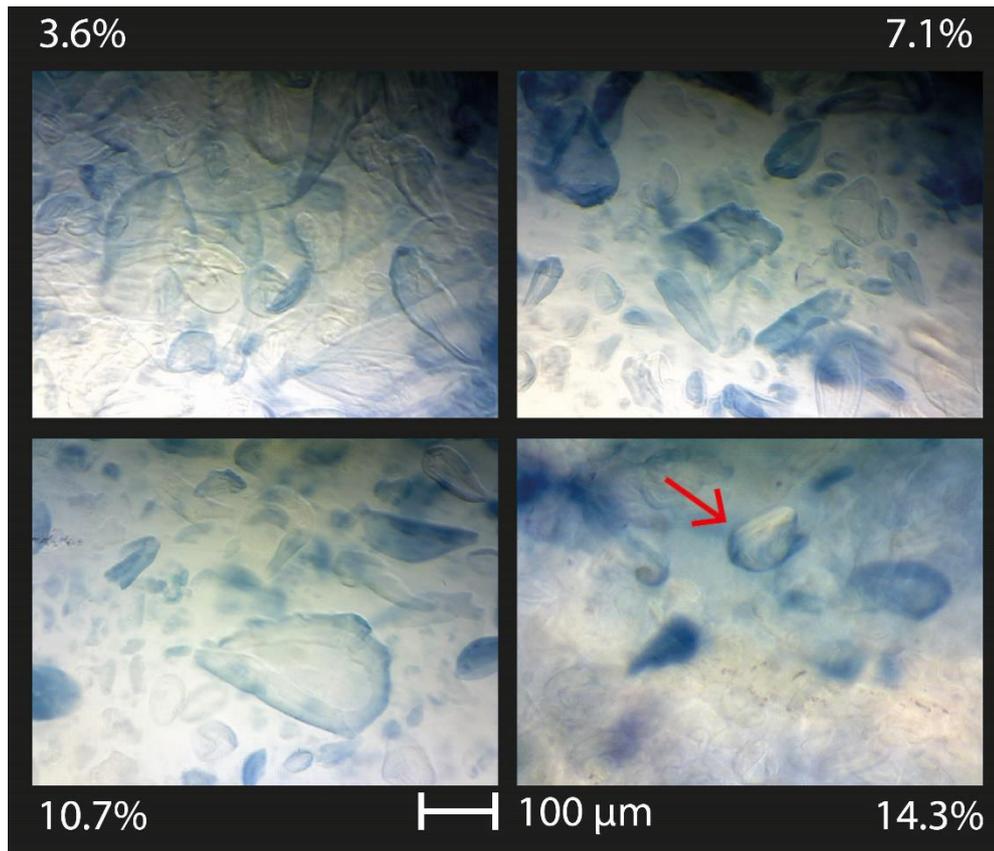


Figure S1: Light microscopy* images of the potato starch gel at different starch concentrations (3.6, 7.1, 10.7 and 14.3%) after the RVA analysis using the standard temperature protocol (50-95-50°C). The white line measures 100 µm. The red arrow indicates an incompletely gelatinized granule.

* Light microscope (model L1000, Bioval, Curitiba, Brazil) with a 20 W halogen lamp. Lugol solution (1% I₂ and 2% KI) was used to better visualize the gels. The magnification was of 400x and a portable camera of 1.3 megapixels was used to obtain the images.

APPENDIX C: Starch modification by ozone: correlating molecular structure and gel properties in different sources

22 **Abstract:**

23 Cassava, corn (maize) and potato starches were subjected to ozone treatment at
24 different processing times. The three sources were processed under the same
25 conditions, to allow comparisons. In order to prove the ozone treatment effectiveness
26 and versatility, both molecular structure and functional properties of treated products
27 were evaluated. The molecular structure of the starch samples, analyzed using high-
28 performance size-exclusion chromatography and gel permeation chromatography,
29 proved the molecular depolymerization caused by the ozone treatment. Similarly, the
30 starch properties were also affected by ozonation, presenting in general a decrease of
31 the samples' apparent viscosity during their pasting and a decrease of the complex shear
32 modulus (G^*) of the gels immediately formed with the treated starches. Moreover, the
33 same gels were evaluated after cooling and storage, and the ozonation showed to
34 increase or decrease their strength, depending on the employed starch concentration.
35 All the results illustrated relationships between ozonation conditions, starch source and
36 analysis conditions (especially starch concentration), as well as their influence on the
37 samples' behavior and functional properties. Results for cassava and potato starches
38 were similar, while corn starch presented an opposite behavior. In conclusion, ozone
39 technology proved to be a highly efficient and versatile technology with potential
40 industrial application and with the capacity of providing functional properties to starches
41 used for food formulations.

42

43 **Keywords:** chemical modification; starch source; ozone; emerging technologies; maize;
44 tapioca

45 **1. Introduction**

46 Starch modification is widely used to improve the functional properties of starches
47 from different sources in different industries, such as food, paper, chemical,
48 petrochemical, pharmaceutical, textile, etc. Although being widely applied, chemical
49 modifications usually lead to highly polluted wastes (Kaur, Ariffin, Bhat, & Karim, 2012;
50 Liu, Weber, Currie, & Yada, 2003), what goes against the increasing consumer's demand
51 for sustainable products that have low environmental and social impacts (Duncan, 2011;
52 Shankar & Rhim, 2016).

53 To work around this issue, some alternatives for those chemical processes are
54 being considered. Ozonation is one of the most promising techniques, which is gaining
55 attention due to many positive aspects of ozone, such as being highly oxidative, easily
56 generated and environmentally friendly, since it decomposes to oxygen (Çatal &
57 İbanoğlu, 2014; Mahapatra, Muthukumarappan, & Julson, 2005).

58 In fact, the starch ozonation technique was previously described and evaluated
59 by different authors (Castanha, Matta Junior, & Augusto, 2017; Çatal & İbanoğlu, 2014;
60 Chan et al., 2011; Klein et al., 2014; Oladebeye, Oshodi, Amoo, & Karim, 2018). Besides,
61 this technology has shown potential to improve the functional properties of different
62 starches. For instance, for cassava starch, ozonation was able to improve the oven
63 expansion properties (Matta Junior, Castanha, Anjos, Augusto, & Sarmiento, 2019), 3D
64 printability (Maniglia et al., 2019) and to produce biodegradable plastics with enhanced
65 properties (La Fuente, de Souza, Tadini, & Augusto, 2019). Among those key properties,
66 the unique characteristics of gels obtained from ozone modified starches are highlighted,
67 illustrating that the gel properties are, usually, important functional properties for further
68 applications. However, most of the mentioned studies were based on a single starch
69 source. Moreover, it is impossible to direct compare the different studies, when the
70 applied ozonation conditions differ widely. Furthermore, the literature in general presents
71 the properties of the obtained pastes or gels considering only one approach. Finally,
72 direct correlations between effects of processing in different starch sources, combined

73 with the modified starches' molecular structures and the properties of gels produced with
74 the modified starches, are rarely addressed in the literature, especially under non-
75 standard conditions of analysis. Consequently, many of the reported results are
76 contradictory.

77 In this work, the same ozonation conditions were used to modify cassava, corn
78 (maize) and potato starch samples. The starches' structure (molecular size and
79 macromolecular features) and properties of products containing those starches (i.e.
80 paste and gel characteristics) were evaluated and correlated to the processing
81 conditions. Therefore, the main objective of this work was to evaluate the ozone
82 processing of different starches, to analyze their molecular structures and to relate them
83 with the properties of pastes and gels obtained with the treated starches, especially
84 considering functional properties for further applications.

85

86 **2. Material and methods**

87

88 *2.1. Starch samples and processing conditions*

89 Native cassava starch (Amilogill 1500) was kindly provided by "Cargill,
90 Incorporated" (Campinas, Brazil). Native corn (maize) starch (Argo CS 3400) was kindly
91 provided by "Ingredion Brasil Ingredientes Ltda" (Mogi Guaçu, Brazil). Native potato
92 starch was isolated from tubers of *Solanum tuberosum* L. (Monalisa cultivar), being
93 extracted and characterized according to the described in Castanha et al. (2017). All
94 starch samples presented a purity > 99%. The starch apparent amylose content was
95 evaluated according to the ISO methodology (1987), based on the blue value method,
96 being characterized as approx. 29% (native cassava starch), 31% (native corn starch)
97 and 28% (native potato starch).

98 The three starch sources were modified using the same ozonation conditions,
99 thus allowing a direct comparison among them. The ozone treatment was performed as
100 described in Castanha et al. (2017), aiming to better understand the effect of the same

101 ozone treatment on starches with different structures. In short, 10% starch slurries (70 g
102 of starch + 630 mL of water at 25 °C) were ozonated for 15 and 30 min, in a glass reactor
103 (56 cm of height and 6 cm of internal diameter). Industrial oxygen (95% purity) was
104 inserted into an ozone generator unit (Ozone & Life, O&G Model RM 3.0, São Jose dos
105 Campos, Brazil), and the ozone gas produced was bubbled into the starch suspension.
106 The gas flow in the reactor was maintained constant at 1 L/min and the ozone
107 concentration in the gas current was approx. 46 mg O₃/ L (measured by an ozone
108 monitor, Model 106-H, 2B Technologies, Boulder, USA). After processing, the distilled
109 water was removed through decantation and centrifugation, and the samples were dried
110 (at 35 °C until approx. 12% of moisture content) and sieved (250 µm).

111

112 2.2. *Starch molecular evaluation*

113

114 2.2.1. *Molecular size distribution profile using high-performance size-exclusion 115 chromatography system (HPSEC), with multiangle laser light scattering (MALLS) 116 and differential refractive index (RI) detectors*

117 The molecular size distribution profiles were evaluated using chromatographic
118 analysis. The samples' preparation was performed according to the described by Zhang,
119 Ao & Hamaker (2006), with minor modifications. In detail, 100 mg of starch were
120 dispersed in 90% dimethyl sulfoxide (DMSO) and heated in a boiling water bath for 1 h
121 with constant stirring. After their complete dissolution, the samples were slowly stirred
122 overnight (for about 12 h) at room temperature. Then, the starch was precipitated using
123 absolute ethanol and the supernatant was separated and removed using centrifugation.
124 The samples were repeatedly washed with ethanol to eliminate traces of DMSO. The
125 obtained samples were then vacuum-dried at 50 °C and stored in a refrigerator until
126 further use. Before the injection into the chromatographic column, the dried samples
127 were suspended in boiling distilled water (at a concentration of 3 mg/ mL) and heated in
128 a boiling water bath for 30 min, being then slowly stirred at approx. 50 °C until their

129 complete solubilization. 100 μ L of the obtained starch solution were then filtered (5 μ m)
130 and injected in a high-performance size-exclusion chromatography system (HPSEC),
131 using deionized water with 0.02% NaN₃ as mobile phase, at a flow rate of 1.3 mL/min.
132 The system was equipped with a Sephacryl S-500HR column (Amersham Bioscience,
133 Piscataway, NJ, USA), a pump (model LC-10AT vp, Shimadzu Corp., Columbia, MD,
134 USA) and a syringe sample loading injector (model 7125, Rheodyne Inc., Cotati, CA,
135 USA). The column was coupled to a multiangle laser light scattering (MALLS, HELEOS
136 II, Wyatt Technology, Santa Barbara, CA, USA) and a differential refractive index (RI,
137 Optilab rEX, Wyatt Technology, Santa Barbara, CA, USA) detectors (HPSEC-MALLS-
138 RI). The scattering data (MALLS results) were used to perform the calculations of the
139 macromolecular characteristics of the samples using the ASTRA software (Version
140 5.3.4.20, Wyatt Technology, Santa Barbara, CA, USA). A monomodal distribution was
141 considered, and the Berry method and a refractive index increment (dn/dc) of 0.146 g/mL
142 were selected based on literature information for similar systems (Bello-Perez, Agama-
143 Acevedo, Lopez-Silva, & Alvarez-Ramirez, 2019; Zhang et al., 2006).

144

145 *2.2.2. Molecular size distribution using gel permeation chromatography (GPC) based* 146 *on iodine affinity (blue value – BV)*

147 The molecular size distribution profiles based on the samples' iodine affinity were
148 evaluated using gel permeation chromatography (GPC), described in detail by Castanha
149 et al. (2017). The samples were prepared using similar conditions as described by the
150 authors: gelatinized in DMSO, precipitated using ethanol and stored in a refrigerator until
151 further use. Before the injection, the stored samples were completely solubilized in
152 distilled water (at a concentration of 3 mg/ mL). Then, 4 mL of the obtained solution were
153 filtered (5 μ m) and injected into the column (2.6 cm diameter and 70 cm high) packed
154 with Sepharose CL-2B gel (Sigma, Sweden) at a flow rate of 1.0 mL/min. A solution of
155 NaCl (25 mmol/L) and NaOH (1 mmol/L) in distilled water was used as mobile phase.
156 The system ended in a fraction collector (Gilson model FC203B, Middleton, England),

157 programmed to collect 4 mL of mobile phase/tube. Each tube was then colored using
158 iodine solution (1% I₂ and 2% KI) and analyzed using the blue value (BV) method
159 described by Juliano (1971), at 630 nm wavelength, in a spectrophotometer (Femto,
160 Model 600S, São Paulo, Brazil).

161

162 2.3. *Granule morphology*

163 The morphology of the native and modified starch granules, before their
164 gelatinization, was evaluated using a light microscope (model L1000, Bioval, Curitiba,
165 Brazil) with a 20 W halogen lamp. A polarizing filter was used to identify starches' Maltese
166 cross. The magnification was of 400x and a portable camera of 5 megapixels was used
167 to obtain the images.

168

169 2.4. *Gel formation and pasting properties*

170 Starch gels were obtained using a Rapid Visco Analyzer equipment (RVA-4,
171 Newport Scientific Pvt. Ltd., Warriewood, Australia), and pasting properties were
172 evaluated with the Thermocline software (version 3.0, Newport Scientific Pvt. Ltd.,
173 Warriewood, Australia).

174 Two different starch/ water proportions were used. The first proportion was 3 g of
175 starch mixed with 25 g of distilled water (10.7% of starch), being the standard
176 concentration of the RVA analysis. The second proportion was 1.4 g of starch mixed with
177 26.6 g of distilled water (5% of starch), being a non-standard concentration of analysis,
178 but a concentration similar to those generally used in the food industry. In both cases,
179 the starch mass was corrected to a 14% moisture basis.

180 After adding water, the samples were immediately evaluated using the "Standard
181 2" program, which consists in heating the samples until 50 °C, at constant stirring, then
182 held at 50 °C for 1 min, heating to 95 °C at 6 °C/min, held at 95 °C for 5 min, cooled to
183 50 °C at 6 °C/min, and held at 50 °C for 2 min. The pasting properties were obtained
184 from this procedure.

185

186 2.5. *Visible characteristics of the starch pastes*

187 The visible aspect of the starch pastes (at 10.7%) immediately after the RVA
188 analysis were evaluated through light microscopy (model L1000, Bioval, Curitiba, Brazil,
189 with a 20 W halogen lamp). Lugol solution (1% I₂ and 2% KI) was used to better identify
190 the starch molecules within the pastes. The magnification was of 400x and a portable
191 camera of 1.3 megapixels was used to obtain the images.

192

193 2.6. *Viscoelastic properties of the gels*

194 Starch dispersions (5% m/m) were gelatinized using an RVA equipment, as
195 previously described. The obtained gels were maintained inside a water bath at 25 °C
196 and evaluated within 1 h of preparation, being the gel here named “gel-1h”. The rheology
197 of those recently formed gels were then evaluated through their viscoelastic properties.

198 The rheological analysis was carried out using a Discovery-3 rheometer (DHR3,
199 TA Instruments Ltd., New Castle, DE, USA), with an aluminum plate-plate geometry (40
200 mm diameter, 1000 µm gap). The samples were carefully transferred to the Peltier Plate
201 (at 25 °C), trimmed after geometry setting and then left at rest for 300 s. Water loss was
202 minimized by using a solvent trap. Different steps were carried out.

203 Firstly, the extent of the viscoelastic region was determined performing an
204 oscillation stress sweep ranging from 0.1 to 10 Pa, at 25 °C and 1 Hz of frequency.
205 According to the obtained results, a stress value of 1 Pa was used to be within the linear
206 viscoelastic region of the gels.

207 Then, the gel viscoelastic properties were evaluated using a frequency sweep
208 test, in which the samples were subjected to a stress of 1 Pa at 25 °C, and the frequency
209 sweep ranged from 0.1 to 10 Hz. The storage, or elastic modulus (G'), and the loss, or
210 viscous modulus (G''), were evaluated over the oscillatory frequency (ω). Both storage
211 (G') and loss (G'') moduli were fitted by a power function of the oscillatory frequency (ω)
212 given by Equations 1 and 2 (Rao, 1999); and the coefficients k', k'', n' and n'' were

213 calculated. The complex shear modulus (G^* , equation 3) of the samples was also
214 calculated.

215

$$216 \quad G' = k' \cdot \omega^{n'} \quad (\text{Equation 1})$$

$$217 \quad G'' = k'' \cdot \omega^{n''} \quad (\text{Equation 2})$$

$$218 \quad G^* = (G'^2 + G''^2)^{0.5} \quad (\text{Equation 3})$$

219

220 Finally, the mechanical behavior of the gels-1h under heating was evaluated, in
221 order to verify if their gelatinization after the RVA treatment was complete. For this
222 analysis, a temperature sweep procedure was used. New gel-1h samples were
223 subjected to constant frequency (1 Hz) and stress (1 Pa), while the temperature was
224 increased from 5 to 95 °C, at a rate of 5 °C/ min. The complex shear modulus (G^* ,
225 equation 3) changes over the increasing heating were monitored.

226

227 2.7. *Gel texture after cooling and storage*

228 The rheology of partially retrograded gels was evaluated through their texture.

229 After each RVA test previously described (using both 5 and 10.7% of starch
230 concentration), the pastes were stored in 40 x 20 mm (diameter x height) plastic cups for
231 24 h, to promote their partial retrogradation. To ensure uniform temperature and moisture
232 of the samples before the analysis, the cups were maintained inside a desiccator with
233 water at the bottom, and the desiccator was kept inside a refrigerator at a temperature
234 of 5 ± 2 °C. This gel is here named “gel-24h”.

235 The texture of the gels-24h was evaluated through a puncture test using a
236 Texture Analyzer (TA.XT Plus, Stable Micro Systems Ltd., Surrey, UK) with a load cell
237 of 50 kgf (490 N).

238 After conditioning, the samples were uniaxially perforated using a 0.5 cm
239 cylindrical probe (P/0.5R) at 1 mm/s, until the distance of 13 mm. The force measured

240 by the equipment as a function of the penetration depth was evaluated to obtain the gel
241 strength.

242

243 2.8. *Experimental design and statistics*

244 Each process was performed in triplicate and the analyses were performed at
245 least in duplicate for each sample. The averages and the standard deviations were
246 calculated and, when appropriate, the Tukey's multiple comparisons was used. A
247 significance level of 5% was considered. Non-modified samples (mentioned as "native"
248 or "0 min") were used for comparisons.

249 Principal Components Analysis (PCA) was performed to discriminate the
250 starches samples (native and processed) according their main properties. The analysis
251 enables the reduction of the dependent variables (dimensions) to only 3 dimensions to
252 improve the interpretation of results. For this analysis, the Correlation Circle and the
253 Observation Chart were joined in one plot for better interpretation. This analysis was
254 performed using Statistica 12.0 software (StatSoft, USA).

255

256 **3. Results and discussion**

257

258 3.1. *Starch molecular structure*

259 The molecular size distribution of the native and treated starches is shown in
260 Figure 1, where the progression of the differential refractive index (RI) signal with the
261 elution time and of the blue value (BV) measured on the collected fractions on each
262 sample are plotted. For both analyses (RI and BV), the starch molecules were separated
263 mainly by their sizes. Therefore, the first peak represents the bigger molecules (usually
264 amylopectin), which elute first, while the second peak represents the smaller molecules
265 (usually amylose) (Hizukuri, Abe, & Hanashiro, 2006).

266 By evaluating the general aspect of the RI and BV curves (Figure 1), it is possible
267 to see a common tendency for the three starch sources: the first peak ("amylopectin

268 region”) of the 0-min samples decreased with increasing ozone treatment time, resulting
269 in smaller-sized molecules (eluting in the “amylose region”). Moreover, not only the
270 amylopectin but also the amylose molecules were affected, what is demonstrated by the
271 second peak (eluting later, i.e., a “displacement to the right”). These results indicate a
272 molecular depolymerization due to the ozone action, which is commonly reported in the
273 literature on starch treated with ozone (Castanha et al., 2017; Chan et al., 2011; Maniglia
274 et al., 2019; Oladebeye, Oshodi, Amoo, & Karim, 2013).

275 For cassava starch, after 15 min treatment, virtually all the first peak was
276 dissipated. On the other hand, for potato and, especially, for corn starch, this result was
277 only observed after 30 min of treatment. In other words, considering the molecular
278 depolymerization results, corn starch followed by potato starch were more “resistant” to
279 the ozone treatment and required a longer treatment time to be affected. In turn, cassava
280 starch was depolymerized in a relative shorter time. Those results are in accordance with
281 the other analysis presented in this work, and our main hypothesis for this behavior is
282 related with the intrinsic characteristics of each native starch source, which will be
283 discussed further in this section.

284 The macromolecular characteristics of the starch samples are illustrated in Table
285 1. Considering the 0-min (non-modified) starches, their respective molar masses (M_w)
286 and gyration radius (R_z) (approx. $2.0 \cdot 10^8$ g/mol and 174.1 nm for cassava; $2.1 \cdot 10^8$ g/mol
287 and 178.4 nm for corn; and $5.5 \cdot 10^7$ g/mol and 104.3 for potato) were similar to values
288 found in the literature for cassava: $2.16 \cdot 10^8$ g/mol and 271 nm (Boonna, Rolland-Sabaté,
289 Lourdin, & Tongta, 2019); normal corn: $1.4 \cdot 10^8$ g/mol and 178 nm (Bello-Perez et al.,
290 2019); and potato: $5.4 \cdot 10^7$ g/mol and 149 nm (Bello-Pérez, Roger, Baud, & Colonna,
291 1998). The differences among the starch sources can be related to their molecular
292 chains’ conformation, especially regarding their branching characteristics (Hanashiro,
293 Abe, & Hizukuri, 1996). In fact, different works (Bello-Perez et al., 2019; Bello-Pérez et
294 al., 1998) demonstrated that native starches with a higher percentage of branch linkages
295 had lower M_w , which is the case of the potato starch.

296 All the starch sources exhibited a decrease in their measured M_w and R_z with
297 increasing ozonation time, when compared to the 0-min samples. These results can be
298 attributed to the depolymerization of the starch molecules, especially amylopectin
299 chains, being in accordance with Figure 1 and with what has been reported in the
300 literature (Bello-Pérez et al., 1998; Chan et al., 2011). It is important to mention that,
301 once again, corn starch presented a different behavior when compared to the other
302 starch sources, showing a significant difference between the samples treated for 15 and
303 the 30-min. This behavior was not observed for cassava and potato starches (which
304 showed statistically similar molar mass for samples treated for 15 and 30-min) as also
305 illustrated in Figure 1.

306 Values of \bar{D} indicate the broadness of the molar mass distribution, a perfectly
307 uniform polymer should present a value of $\bar{D} = 1$ (Gilbert et al., 2009). Therefore, the
308 molecules of the control samples (0-min) could be described as highly uniform, since
309 their \bar{D} values were close to 1 (Table 1), especially cassava starch ($\bar{D} = 1.1$). On the
310 other hand, ozonated samples had values of $\bar{D} > 1.5$, indicating that the mass distribution
311 was affected by the ozone treatment by turning the molecules less homogeneous (Table
312 1). The potato starch presented an increasing \bar{D} value after 15 min of treatment, which
313 decreased again after 30 min. The cassava starch presented a similar pattern; however,
314 the 30-min cassava sample was not significantly different from the 0 and 15-min. This
315 behavior can be explained considering the progress of the ozonation reaction: it starts
316 by generating molecular species of different sizes (higher \bar{D} values) by randomly
317 hydrolyzing their bonds. However, with the advance of the treatment, the molecular
318 distribution becomes homogeneous again (smaller \bar{D} values), indicating that the
319 molecules were hydrolyzed to the point of presenting similar smaller sizes. Considering
320 this information, it is possible to say that the corn starch reaction progress is slower than
321 the other starches under the same process conditions, since its \bar{D} value is still increasing
322 with ozonation time, indicating that this sample did not reached the phase of a “molecular
323 distribution homogeneity” observed for the other starch samples. Similar results (i.e.

324 higher D values after ozonation) were found in the literature for pigeon pea, lima bean
325 and jack bean starches (Oladebeye et al., 2018) and for corn and sago starches (Chan
326 et al., 2011), which were related to the molecular depolymerization undergone by those
327 samples.

328 In summary, these results (Figure 1, Table 1) indicate that ozone processing led
329 to a molecular depolymerization of all native samples. The main difference between the
330 starch sources was related to the apparent “resistance” of the corn starch to the ozone
331 action, taking a longer time to be affected if compared to the cassava and potato
332 starches. In order to explain the behavior of corn starch during ozonation our hypothesis
333 is that minor components such as lipids naturally present in corn starch granules are able
334 to form amylose-lipid complexes (Zobel, 1988). These complexed lipids are known to
335 affect several starch properties and functionality, such as the reduction of swelling power
336 (Tester & Morrison, 1990) and the increase of the starch gelatinization temperature (Jane
337 et al., 1999). These characteristics were observed in the pasting behavior of the starches
338 and are further discussed. The evaluation of ozonation impact on gel properties was,
339 thus, evaluated.

340

341 3.2. *Starch granular structure*

342 Figure 2 illustrates the morphology of the starch granules. It is possible to observe
343 that the ozone treatment had no apparent influence on the visual aspect or crystallinity
344 (qualitatively analyzed through polarized light microscopy) of the granules. It is important
345 to emphasize that native cassava and corn starch granules are highly heterogeneous
346 and, therefore, it is very difficult to observe any changes in their structure, especially
347 using light microscopy. On the other hand, native potato starch granules exhibit a smooth
348 surface and regular oval shapes, so changes are easily detected. Nevertheless, no
349 significant changes are observed after the ozonation for the starch samples.

350

351 3.3. *Gel formation and pasting properties*

352 The pasting properties of the different native starches and after the ozone
353 treatments for two different starch concentrations (5% and 10.7%), are illustrated in
354 Figure 3, and their main pasting parameters are reported in Table 2.

355 Before discussing the results, it is important to mention that increases in apparent
356 viscosity (μ_{app}) during starch pasting represents the rheological interactions between the
357 dispersed swollen granules with the continuous phase and with themselves, while the
358 peak μ_{app} indicates the thickening power of a starch dispersion. At high temperatures
359 with continued shear, the granules are usually torn apart, and the remaining “ghosts”
360 become responsible for the subsequent μ_{app} behavior (Biliaderis, 2009). Therefore, the
361 volume and the rigidity/ deformability of the granules and/or ghosts, as well as their
362 interactions with other granules and with the continuous phase, plays a more important
363 role than the molecular characteristics of the samples regarding the pasting properties.

364 At 5%, the pasting properties of the different starch sources were similar. This
365 result indicates that the continuous phase in that case (less concentrated system) had a
366 higher influence on the pasting results than the granules' characteristics. In contrast, for
367 the samples with concentration 10.7% the pasting properties were highly influenced by
368 the starch type (Figure 3, Table 2). Considering the peak μ_{app} results (Table 2), the 0 and
369 15-min cassava starch samples had similar results ($p < 0.05$), while the 30-min sample
370 was comparatively lower. For the potato starch, the peak μ_{app} decreased gradually with
371 ozonation time, whereas in contrast for corn starch the peak μ_{app} continuously increased
372 with ozonation time. Regarding the trough and the final μ_{app} results (Table 2), cassava
373 and potato starches presented similar patterns (gradual decrease with ozonation time),
374 and, in general, the μ_{app} values were negligible (i.e. below the threshold for reliable RVA
375 measurement). Unlike the other two samples, corn starch exhibited a “milder” decrease
376 of the trough and final μ_{app} (Figure 3). In fact, the trough μ_{app} of the 30-min ozonated corn
377 starch (approx. 377 mPa·s) was higher than the trough μ_{app} values exhibited by all
378 ozonated potato and cassava starches. Finally, considering the samples' pasting

379 temperatures (Table 2), each sample exhibited a distinct behavior with ozonation time:
380 the pasting temperature increased for cassava starch (from approx. 66 °C to 71 °C after
381 30 min), decreased for corn starch (from approx. 75 °C to 73 °C after 30 min) and
382 presented no variation for potato starch (showing, however, a slightly increase after 30
383 min - from 70 to 72 °C - although without statistical proof).

384 Diverging results of pasting properties for ozonated starches were reported in the
385 literature by different authors, especially regarding the peak μ_{app} and pasting
386 temperatures (Castanha et al., 2017; Castanha et al., 2018; Çatal & İbanoğlu, 2012,
387 2014; Chan, Bhat, & Karim, 2009; Chan et al., 2011; Klein et al., 2014; Oladebeye et al.,
388 2013). It must be considered, however, that different starch sources and ozone
389 processing conditions were used in the mentioned works, which makes the comparison
390 impossible.

391 Light microscopy and rheometry (Figures 4 and 5) showed incomplete
392 gelatinization of corn starch after RVA and relatively more complete gelatinization of
393 cassava and potato starches. In Figure 4, it is possible to see some intact granules
394 dispersed in the corn starch paste, while the potato and cassava starches exhibited a
395 more “complete” gelatinization (with ghosts dispersed among a smooth gel network).

396 Figure 5 shows the behavior of the starch pastes subjected to a heating from 5
397 to 95 °C. The expected behavior for a gelatinized starch is those of a polysaccharide
398 dispersion, i.e., the reduction on consistency (measured by G^*) with increase of
399 temperature. This was the behavior of potato and cassava starches. However, corn
400 starch exhibited increasing G^* for the 0 and 15-min (in a lower extent) samples, until a
401 temperature close to 50 °C is reached, indicating that remaining intact granules were still
402 gelatinizing until this temperature, after which G^* started to decrease. In fact, those
403 results can be related to an impaired swelling of the granules during the RVA assay due
404 to its complexed lipid content (Tester & Morrison, 1990), as previously discussed. On the
405 other hand, after the ozone treatment, corn starch exhibited a higher peak μ_{app} , indicating
406 that the ozonation improved its thickening capacity, probably due to a granular

407 weakening that increased the water uptake capacity of the granules, as discussed by
408 Castanha et al (2019).

409 In summary, by gathering this information and the results reported in Figure 3
410 and Table 2, it is possible to conclude that both ozonation treatment and granular
411 characteristics of the starches samples affected their pasting behavior, especially at
412 concentrations of 10.7%. Finally, two other important points regarding the pasting
413 properties are worth highlighting. Firstly, the results proved that evaluating only one
414 starch concentration can be problematic in RVA: as shown in Figure 3, the same samples
415 presented very different trends at 5 and at 10.7%. Therefore, evaluating only one
416 concentration can lead to misinterpretation or even incomplete conclusions about the
417 sample or about the effect of ozone processing. Secondly, at the conditions applied in
418 this work, the characteristics of the samples' structure were as important as the
419 processing conditions to obtain desired and more suitable properties for further food and
420 non-food applications. However, some typical relations, such as the amylose proportion
421 or granule size, alone, cannot describe the ozonation effectiveness.

422

423 3.4. *Viscoelastic properties of the recently formed gels*

424 The viscoelastic properties of the recently formed gels (gel-1h) are shown in
425 Figure 6 (G^* over frequency) and in Figure 7 (consistency coefficients and behavior
426 indexes). For these rheological measurements, the pastes obtained from the RVA tests
427 were evaluated after no more than 1 h. Therefore, there was a partial gel formation, since
428 the sample was not allowed to fully retrograde. Besides, only the pastes at 5% were
429 evaluated, since at 10.7% the pastes become too hard and brittle for this analysis
430 (becoming heterogeneous after cooling - as illustrated in Figure S1, supplementary
431 material).

432 Cassava and potato starches presented very low G^* values after ozonation when
433 compared to the native samples (Figure 6), presenting similar trends despite the
434 difference in the magnitude of the values. In fact, their consistency coefficients (k' , k'' –

435 Figure 7) presented negligible values after ozonation, with the cassava starch having a
436 reduction of approx. 98% in its elastic consistency coefficient (k') after 15 min of
437 ozonation, and with potato starch having a reduction of approx. 99% in k' after the same
438 treatment time. Once again, corn starch exhibited slower modifications in relation to the
439 other sources, showing a reduction of ~53% in k' after 15 min of ozonation, and of ~89%
440 after 30 min. These results are in accordance with the complex moduli values illustrated
441 in Figure 6), as well as with the RVA results for final μ_{app} (Figure 3).

442 The behavior index (n' , n'') of the samples also changed and even indicated a
443 change in the rheological behavior of the samples for some cases. The 0-min cassava
444 starch sample, for example, presented $n'/n'' < 1$, and after 15 min of ozonation values
445 were $n'/n'' > 1$. The 0 and 15-min potato starch samples had values $n'/n'' < 1$, and values
446 $n'/n'' > 1$ after 30 min of ozonation. On the other hand, for corn starch, all n' and n''
447 values were < 1 , indicating no change in its rheological behavior after ozone processing.
448 Chan et al. (2011) and Çatal & İbanoğlu (2013) observed $n'/n'' < 1$ for both control and
449 ozonated starch samples. However, a higher value of n'/n'' was observed after the
450 ozonation, being in accordance with our results.

451

452 3.5. *Gel texture after cooling and storage*

453 The textural properties of starches after cooling and storage at 5 °C for 24 h (gels-
454 24h) are illustrated in Figure 8; samples with concentrations 5 and 10.7% were
455 evaluated. Results show that cooling and time of storage promoted gel formation, mainly
456 due to starch retrogradation.

457 By evaluating the concentration effect, it is possible to see that, as expected, the
458 5% gels presented lower strength values when compared to the more concentrated gels
459 (10.7%). In fact, in some cases, the 5% gels presented negligible strength (especially
460 the 30-min treated samples). Also, at 5% concentration, all the starch sources exhibited
461 similar trends, with decreasing gel strength with increasing ozonation time, which agrees

462 with the pasting results (Figure 3) and viscoelastic properties (Figure 6) under the same
463 concentration.

464 Gel formation has been related with the re-association of the gelatinized starches
465 during storage and cooling, including some complex organization within the remaining
466 swollen granules, amylose that has leached out during the gelatinization process and/or
467 ghosts remaining in the system. Therefore, its strength depends on several factors, as
468 size and proportion of amylose and amylopectin molecules, presence of electronegative
469 groups, amount of ghosts and/or swollen granules, starch concentration, etc. (Liu, 2005;
470 Miles, Morris, Orford, & Ring, 1985) – most of these characteristics are changed during
471 ozonation. Therefore, the negligible penetration forces exhibited by the 5% starch gels
472 are likely to be associated to their low concentration, which does not promote the
473 formation of a strong network leading to a gel.

474 On the other hand, at 10.7%, the gels presented higher strength values
475 (especially the corn and potato starches) when compared to samples with a 5%
476 concentration. Moreover, in general, the ozonated 10.7% starches produced stronger
477 gels after cooling, except the 15-min ozonated cassava starch, which showed a particular
478 behavior, as shown in Figure 8. However, despite of being harder, most of the ozonated
479 samples were more “brittle” when compared to the control samples, being ruptured at
480 smaller penetration depth. These results are important from an application perspective,
481 since stronger gels are very desirable but the gel fragility is a point to consider. For
482 instance, it was demonstrated that stronger hydrogels produced by cassava starch
483 ozonation produced better 3D printing materials (Maniglia et al., 2019).

484 The gel formation in ozonated starches is a complex phenomenon, since different
485 interactions may take place. The molecular depolymerization can lead to stronger or
486 weaker gels, depending on their molecular size and molecular size distribution. The
487 carbonyl and carboxyl groups, arising from hydroxyl oxidation, affect the attractive
488 (carbonyl) or repulsive (carboxyl) interactions among molecules. Once these aspects

489 occur simultaneously during ozonation, the complex relation among them will dictate the
490 final interactions and, thus, the gel strength.

491 Therefore, results of Figure 8 indicate that ozonation promoted those interactions
492 in a way to improve better association after cooling of 10.7% concentration systems,
493 forming stronger gels. The only exception was the 15-min cassava starch sample, which
494 in fact illustrated the variability of the ozone treatment, since by changing the treatment
495 time it is possible to obtain a weaker (15-min sample) or a stronger (30-min sample) gel
496 when compared to the native starch. That is, the ozone proved to be a flexible method
497 to obtain starches with different and even opposite functional properties for industrial
498 applications.

499 Oladebeye et al. (2018) observed softer and more elastic gels after the ozone-
500 oxidation of pigeon pea, lima bean and jack bean starches, and related their results with
501 a lower retrogradation tendency presented by those samples. The difference between
502 their results with the presented in our work may be due to the ozonation condition applied
503 by the authors (who performed a gaseous ozonation), resulting in starches with lower
504 association capacity.

505

506 3.6. *Starch properties: overview*

507 Each rheological analysis performed in this work illustrates a particular
508 characteristic of the starch sources and the influence of processing conditions and starch
509 concentration on these properties.

510 It is important to stress that the interpretation of rheological analyses performed
511 under different conditions is challenging, since each one is majorly influenced by a
512 distinct physical event, thus affecting the final results (Biliaderis, 2009). For example, the
513 pasting properties (RVA analysis, Figure 3, Table 2) are highly influenced by the
514 granules' characteristics, while the gel viscoelastic properties (rheology analysis, Figures
515 6 and 7) and the gel strength (texture analysis, Figure 8) are more influenced by the
516 molecular characteristics of the gelatinized starch. All of them, however, are highly

517 dependent on the starch concentration and analysis conditions (as temperature, shear
518 forces, etc.).

519 In summary, the pasting and viscoelastic properties of the starch gels before
520 retrogradation showed that the ozone processing was able to decrease the consistency,
521 apparent viscosity and G^* of the samples, while the strength of the retrograded gels could
522 be increased after ozonation. In both cases, however, the ozonation conditions and the
523 starch sources dictated the final results. The relationship between the main results
524 obtained in this work with each starch source and ozonation time were correlated through
525 a principal component analysis (PCA – Figure 9), which is discussed in the next section.

526 In any case, those results are of high industrial relevance, since with the same
527 modification process it is possible to obtain samples with low consistency (facilitating
528 some unit operations, as pumping or mixing) and with higher strength after cooling and
529 storage (boosting the texture of products). It is important to highlight, however, that the
530 starch source and concentration are factors that cannot be ignored. Finally, the obtained
531 results are of scientific relevance, because for the first time are presented correlations
532 between molecular structure and gel properties considering different starch sources
533 processed at the exact same ozonation conditions.

534

535 3.7. *Relationship between starch source, processing conditions and observed results*

536 Figure 9 shows the Principal Component Analysis (PCA) applied to native and
537 processed starches. The three first dimensions components (Dim1, Dim2 and Dim3)
538 described, respectively, 58.6%, 19.5% and 12.0% of all the studied depended variables
539 (starch properties and macromolecular features) – a total of 90.1%. Therefore, the
540 combinations of Dim 1 and Dim 2 or Dim 1 and Dim 3 are most suitable to explain the
541 results, considering the highest explained variance. It is important to highlight that the
542 three dimensions were considered to explain which properties characterized the starch
543 samples (native and processed) since the discrimination of some properties or sample
544 cannot be observed in only 2 dimensions. In Figure 9, dots (spheres and squares)

545 represent the samples and, the closer they are, the most similar they are. Regarding the
546 vectors, they indicate the correlation among the starch properties. Two properties are
547 positively correlated when the angle between the vectors are close to 0° ; they are
548 negatively correlated when the angle is close to 180° ; they are not correlated when the
549 angle is close to 90° . Finally, samples placed near to the vectors represents that samples
550 are characterized by these related properties.

551 According to the PCA analysis, the 0-min potato starch was the most different
552 sample, being mostly affected by its pasting properties results. The 3D plot (Figure 9)
553 illustrates this by showing the native potato ($p - 0$ min) sample far from the other
554 samples. On the other hand, n' and n'' parameters from the rheological analysis
555 determines the similarities between ozonated cassava (15 and 30 min) and ozonated
556 potato (30 min) starches, as shown in the Dim 1 - Dim 2 plot (78.1% of explained
557 variance). This same plot also illustrates that native starches from cassava and maize
558 are more characterized by their molecular weight (Mw) and gyration radius (Rz). Another
559 relevant observation from this 2D plot, is that the ozonated starches became more similar
560 with increasing ozonation time, except the corn starch, which behaved slightly differently
561 from the other starches. This agrees with previous discussions, where the corn starch
562 resistance to ozonation was demonstrated. Regarding the gel strength results, they
563 showed to be more related to processed than native starches (properties that are better
564 observed in the Dim 1 – Dim 3 plot, with 70.6% of explained variance). This correlation
565 illustrates the fact that gel strength is one of the most important property changed during
566 ozonation.

567

568 **4. Conclusions**

569 Cassava, corn and potato starches were modified by ozone treatment using the
570 same conditions. The impact of the process conditions (ozonation time) and the physico-
571 chemical characteristics of the starch sources was evaluated on the measured functional
572 properties. With increasing ozonation time, the samples presented increase in molecular

573 depolymerization, which led to changes in their macromolecular characteristics.
574 Although chromatographic analysis showed that ozonation changed the structure of
575 amylose and amylopectin molecules in the three starch sources, corn starch reacted
576 slightly differently than cassava and potato starches. The starch properties were also
577 affected by the ozone processing conditions and exhibited, in general, a decrease of the
578 apparent viscosity (μ_{app}) during pasting, a decrease of the gel complex modulus (G^*) just
579 after gel formation, and a decrease or increase in gel strength, depending on starch
580 concentration, after cooling and storage. In summary, all the results illustrated the
581 influence of the ozonation conditions, the starch source and the analysis conditions (the
582 starch concentration influenced the samples' behavior) on the functional properties.

583 Nevertheless, despite the influence of the starch source and analysis conditions,
584 the ozone proved to be a highly efficient and versatile technology, providing samples
585 with distinct and applicable properties, being relevant for future industrial applications.

586

587 **Abbreviations and nomenclature**

588 HPSEC = high-performance size-exclusion chromatography

589 MALLS = multiangle laser light scattering

590 RI = differential refractive index

591 BV = blue value

592 \bar{D} = molar mass dispersity

593 M_w = mass-average molar mass

594 R_z = gyration radius

595 RVA = rapid visco analyzer

596 μ_{app} = apparent viscosity

597 G^* = complex shear modulus

598 k' and k'' = consistency coefficients

599 n' and n'' = behavior indexes

600 PCA = principal component analysis

601 **Conflict of interest**

602 The authors have declared no conflict of interest.

603

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619

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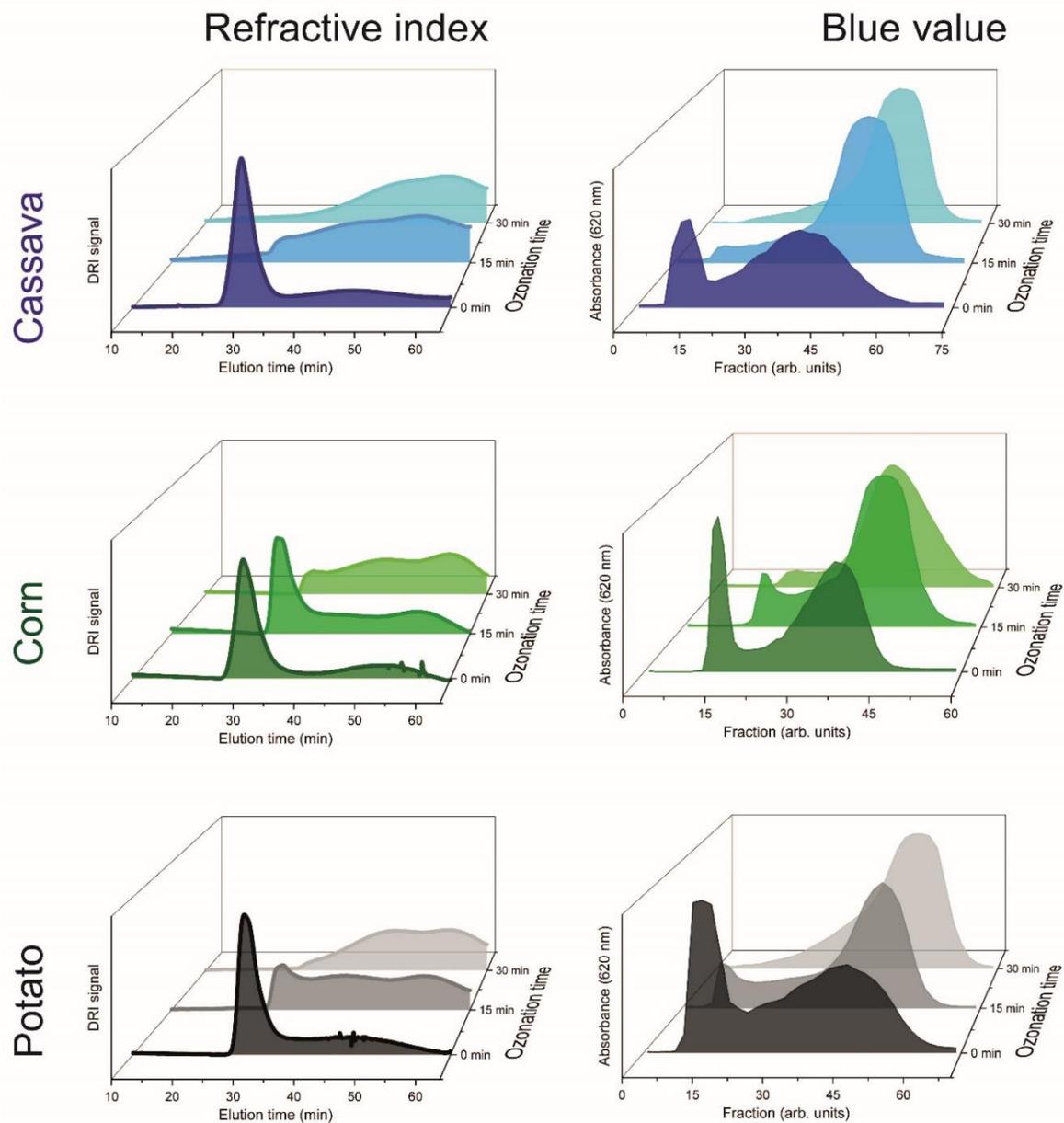


Figure 1. Molecular size distribution profiles of the control and ozonated cassava, corn and potato starches. Left column illustrates the HPSEC profiles based on the samples' differential refractive index (RI) signal. Right column illustrates the gel permeation chromatography profiles based on the samples' blue value (BV) results. Pure glucose was used to mark the end of the starch molecules' RI signal (at 64 min).

Table 1. Molecular characteristics of control and ozonated cassava, corn and potato starches.* The molar mass dispersity (\mathcal{D}) is calculated by the ratio between the mass-average molar mass (M_w) and the number-average molar mass (M_n – data not shown).

Starch sample	Ozonation time	Molar mass - M_w (g/mol)	Gyration radius - R_z (nm)	Molar mass dispersity - \mathcal{D}
Cassava	0 min	$(2.0 \pm 0.4) \cdot 10^8$ ^a	174.1 ± 18.5 ^a	1.1 ± 0.0 ^b
	15 min	$(5.5 \pm 0.2) \cdot 10^6$ ^b	41.7 ± 0.6 ^b	2.0 ± 0.1 ^a
	30 min	$(3.8 \pm 2.2) \cdot 10^6$ ^b	31.5 ± 7.1 ^b	1.6 ± 0.2 ^{ab}
Corn	0 min	$(2.1 \pm 0.0) \cdot 10^8$ ^a	178.4 ± 0.8 ^a	1.2 ± 0.1 ^c
	15 min	$(6.2 \pm 0.4) \cdot 10^7$ ^b	114.7 ± 3.0 ^b	2.5 ± 0.1 ^b
	30 min	$(6.7 \pm 0.4) \cdot 10^6$ ^c	48.9 ± 0.9 ^c	5.1 ± 0.1 ^a
Potato	0 min	$(5.5 \pm 0.7) \cdot 10^7$ ^a	104.3 ± 6.9 ^a	1.4 ± 0.0 ^c
	15 min	$(8.7 \pm 0.9) \cdot 10^6$ ^b	45.2 ± 0.0 ^b	2.5 ± 0.0 ^a
	30 min	$(2.9 \pm 0.3) \cdot 10^6$ ^b	16.7 ± 0.0 ^c	1.6 ± 0.0 ^b

*Average \pm standard deviation. Results followed by the same letter do not differ significantly ($p < 0.05$).

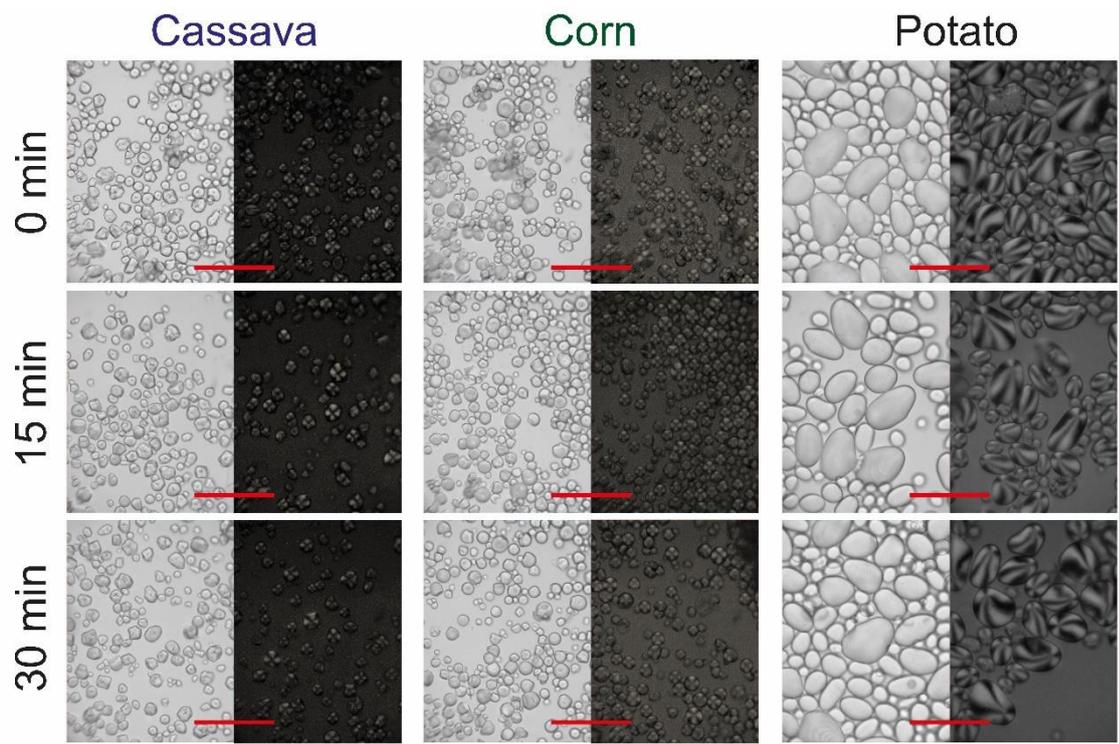


Figure 2. Light microscopy images of the native and ozonated cassava, corn and potato starch granules (non-gelatinized), using both normal and polarized filters. The red line measures 100 µm.

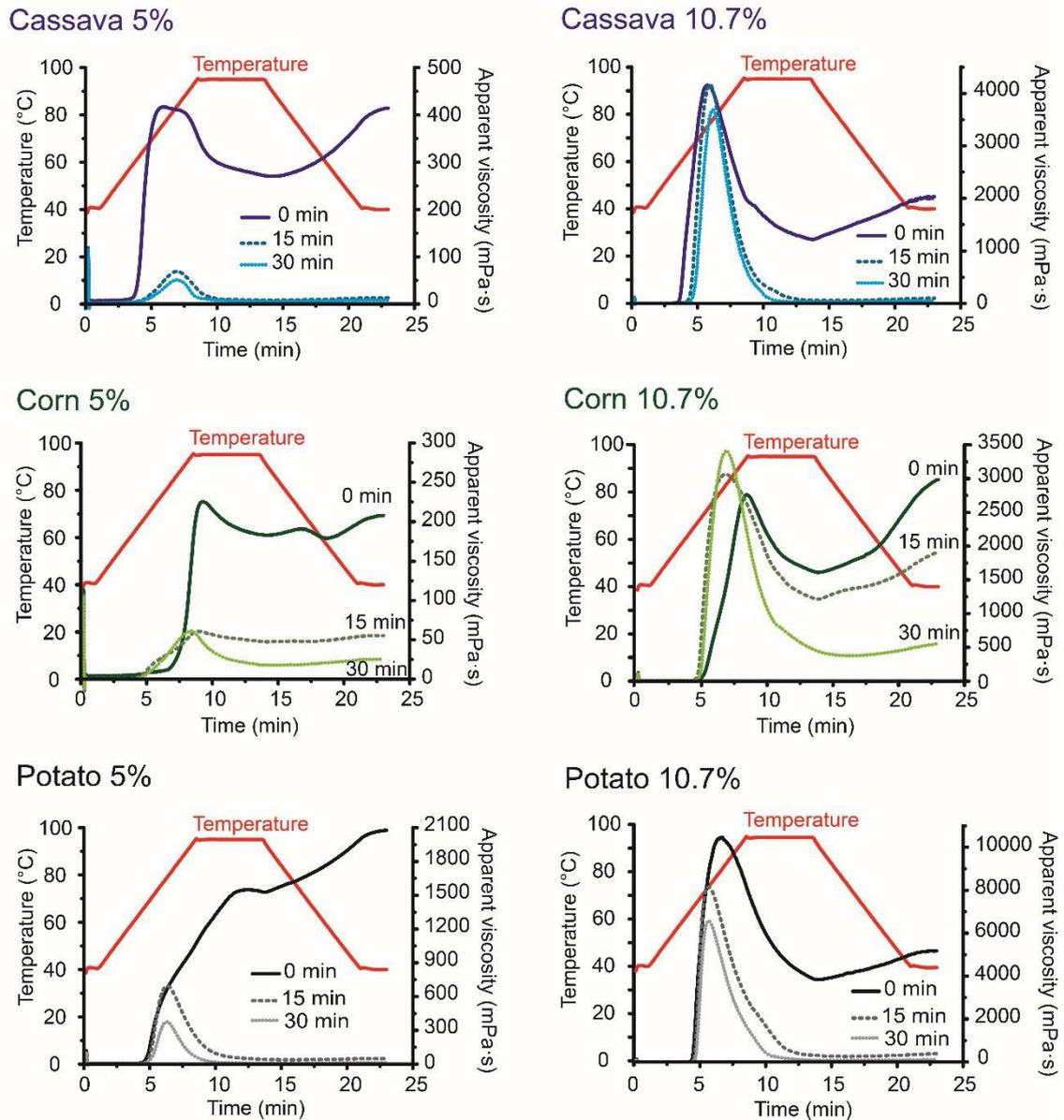


Figure 3. Pasting profiles of the control and ozonated cassava, corn and potato starches. Left column illustrates the RVA profiles at 5% of starch concentration. Right column illustrates the RVA profiles at 10.7% of starch concentration.

Table 2. Pasting parameters of the of the control and ozonated cassava, corn and potato starches at 5 and 10.7% of starch concentration.*

Sample	Ozonation time	Peak μ_{app} (mPa·s)	Trough μ_{app} (mPa·s)	Relative breakdown (%)	Final μ_{app} (mPa·s)	Relative setback (%)	Pasting temperature (°C)
Cassava 5%	0 min	417 ± 4	271 ± 2	35 ± 0	415 ± 2	53 ± 0	69 ± 0
	15 min	69 ± 5	8 ± 1	89 ± 1	13 ± 1	67 ± 13	nd
	30 min	51 ± 2	5 ± 1	90 ± 3	9 ± 2	68 ± 22	nd
Corn 5%	0 min	226 ± 3	179 ± 2	21 ± 0	208 ± 2	16 ± 0	nd
	15 min	61 ± 4	48 ± 4	21 ± 1	55 ± 4	15 ± 1	nd
	30 min	63 ± 6	19 ± 6	71 ± 7	27 ± 7	45 ± 10	nd
Potato 5%	0 min	1550 ± 14	1527 ± 8	1 ± 0	2077 ± 13	36 ± 0	73 ± 1
	15 min	697 ± 66	38 ± 8	95 ± 1	48 ± 8	29 ± 5	74 ± 0
	30 min	374 ± 25	7 ± 1	98 ± 0	12 ± 1	81 ± 17	76 ± 0
Cassava 10.7%	0 min	4158 ± 8	1213 ± 20	71 ± 1	2034 ± 21	68 ± 1	66 ± 0
	15 min	4162 ± 18	55 ± 2	99 ± 0	101 ± 5	83 ± 2	70 ± 0
	30 min	3694 ± 17	30 ± 1	99 ± 0	49 ± 1	67 ± 5	71 ± 0
Corn 10.7%	0 min	2760 ± 30	1612 ± 25	42 ± 0	2985 ± 42	85 ± 0	75 ± 0
	15 min	3066 ± 34	1213 ± 29	60 ± 1	1912 ± 9	58 ± 4	72 ± 0
	30 min	3406 ± 21	377 ± 9	89 ± 0	553 ± 12	47 ± 1	73 ± 1
Potato 10.7%	0 min	10479 ± 34	3833 ± 144	63 ± 1	5175 ± 154	35 ± 1	70 ± 0
	15 min	8161 ± 124	260 ± 24	97 ± 0	383 ± 39	47 ± 3	70 ± 2
	30 min	6570 ± 39	72 ± 3	99 ± 0	104 ± 4	44 ± 1	72 ± 0

* Relative breakdown (%) = [(breakdown/ peak μ_{app})-100]. Relative setback (%) = [(setback/ trough μ_{app})-100]. μ_{app} = apparent viscosity. nd = not detected. Average ± standard deviation. Results followed by the same letter do not differ significantly ($p < 0.05$).

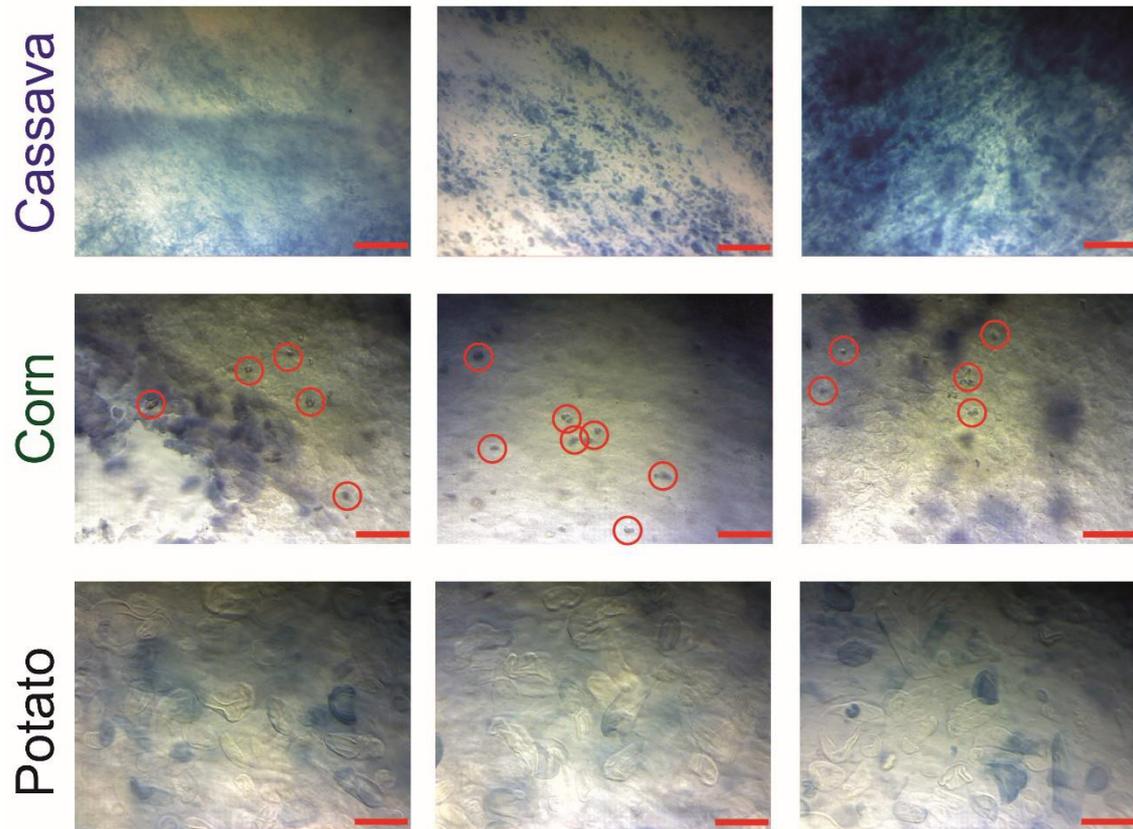
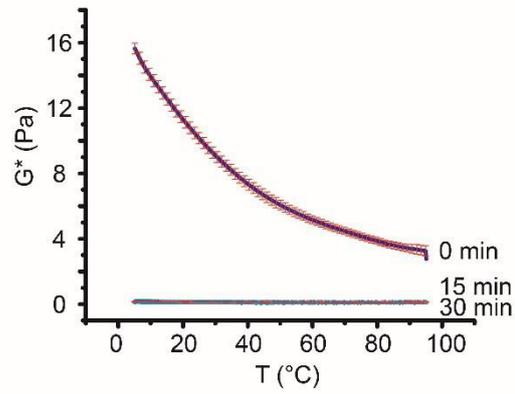
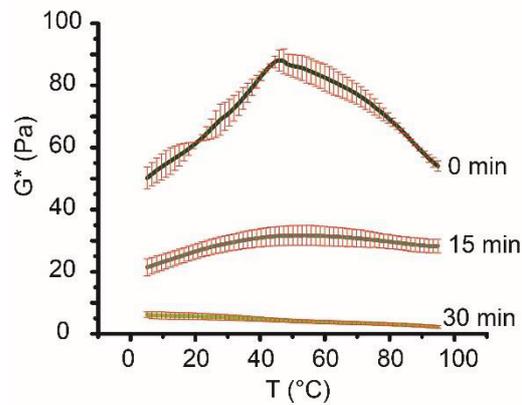


Figure 4. Light microscopy images of native (0 min) cassava, corn and potato starch gels after the RVA analysis for starch concentration of 10.7%. The red line provides a scale of 100 μm . Red circles indicate the non-gelatinized granules. The columns illustrate different regions of the same gel.

Cassava



Corn



Potato

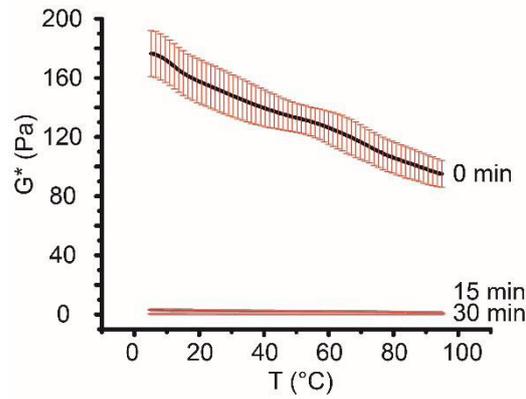
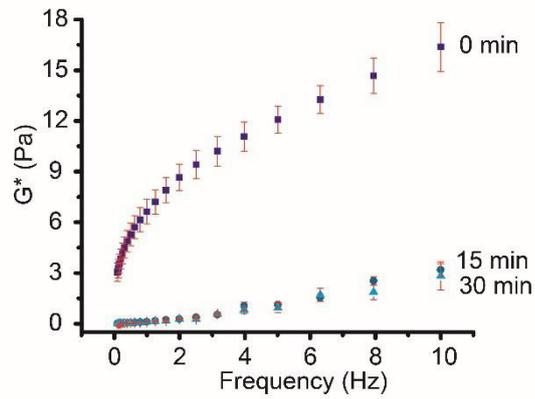
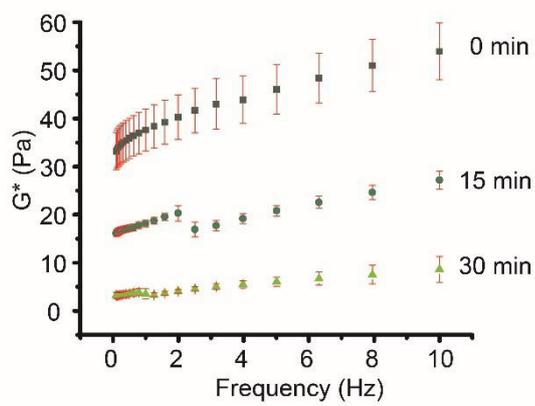


Figure 5. Heating profile of the control and ozonated cassava, corn and potato starch gels (5% of starch concentration, 1 Hz, 1 Pa, $dT/dt = 5^\circ\text{C}/\text{min}$). G^* = Complex modulus. Vertical red bars represent the standard deviations.

Cassava



Corn



Potato

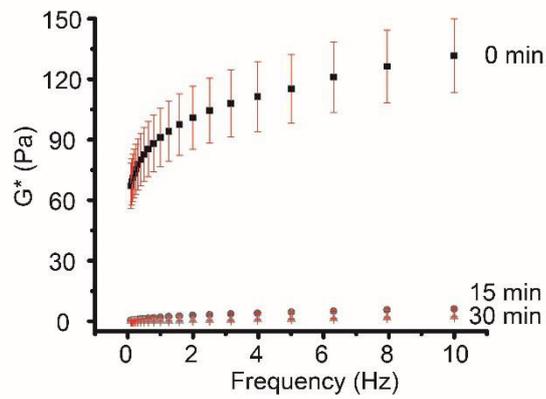


Figure 6. Viscoelastic properties of the control and ozonated cassava, corn and potato starch recently formed gels (gel-1h, 5% of starch concentration, 1 Pa, 25°C). G^* = Complex modulus. Vertical red bars represent the standard deviations.

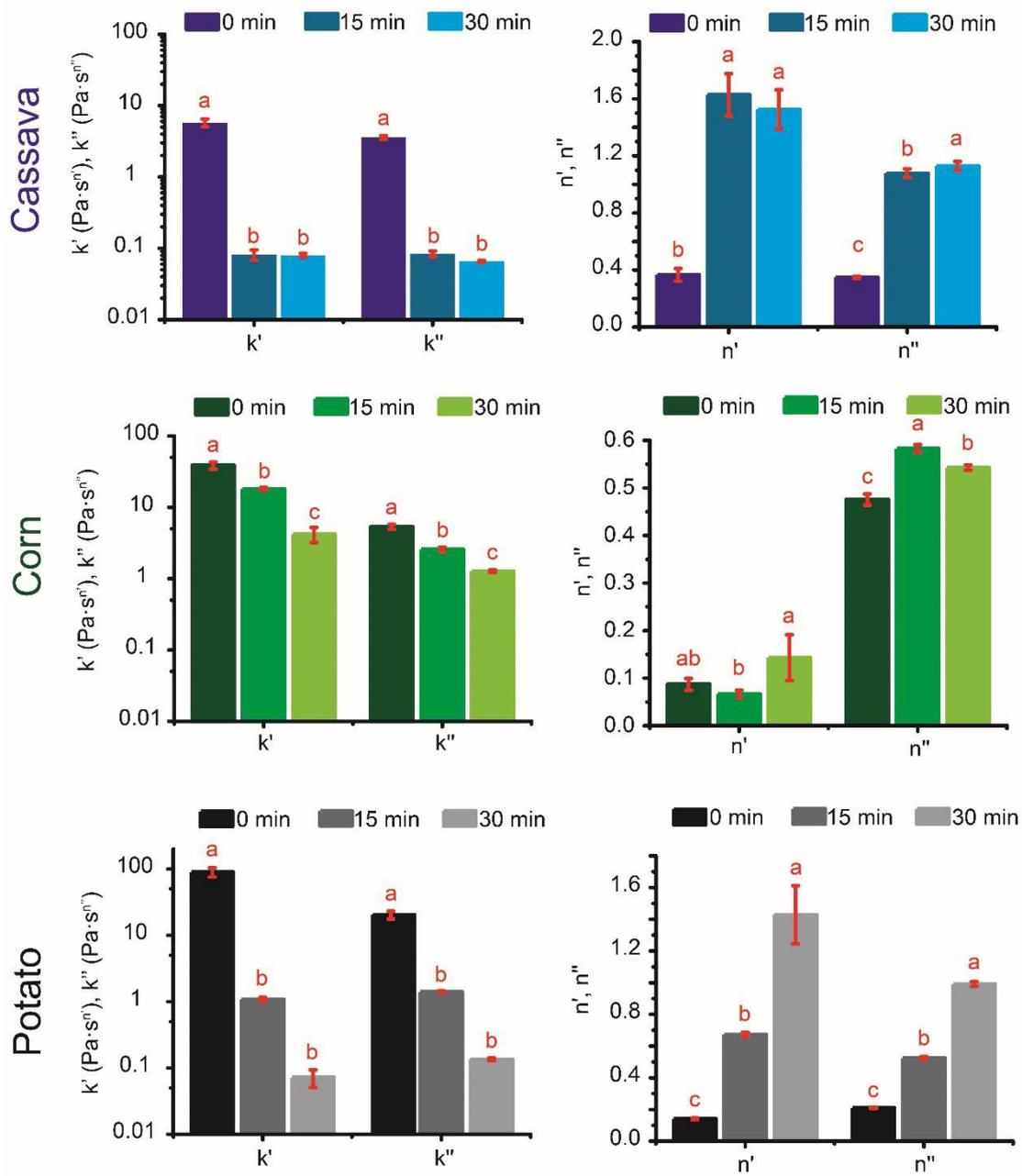
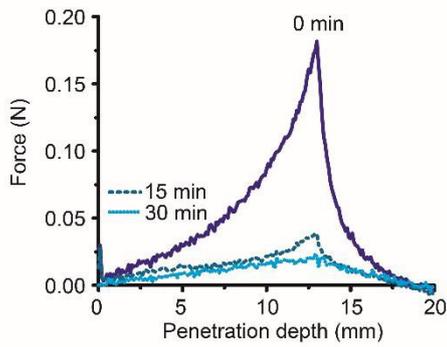
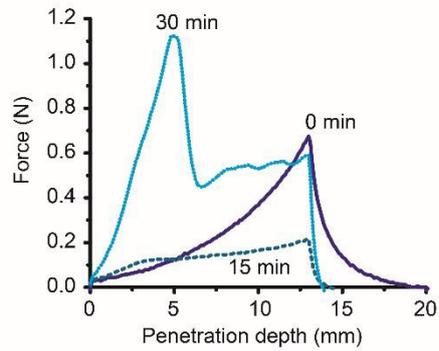


Figure 7. Viscoelastic properties of the control and ozonated cassava, corn and potato starch recently formed gels (gel-1h, 5% of starch concentration, 1 Pa, 25°C). Left column illustrates the elastic and viscous consistency coefficients (k' and k''). Right column illustrates the behavior indexes (n' and n''). Vertical red bars represent the standard deviations. Results followed by the same letter do not differ significantly ($p < 0.05$).

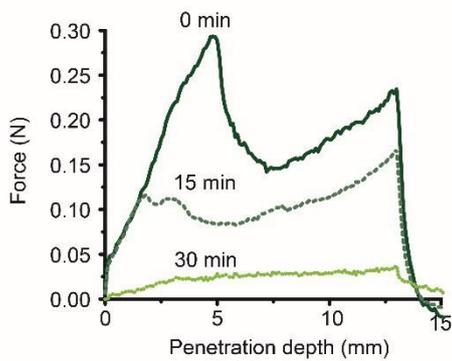
Cassava 5%



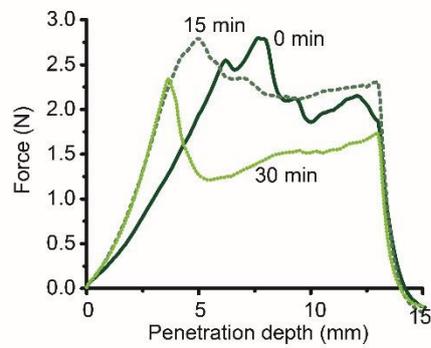
Cassava 10.7%



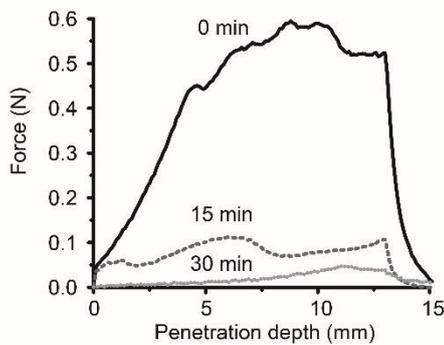
Corn 5%



Corn 10.7%



Potato 5%



Potato 10.7%

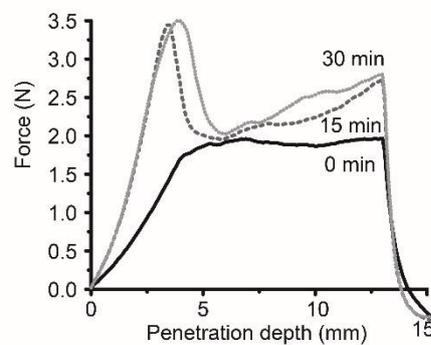


Figure 8. Gel texture of the control and ozonated cassava, corn and potato starches after cooling and storage at 5°C for 24 h. Gels of both 5 and 10.7% of starch concentration are shown. The curves illustrate the strength of each gel-24h until the end of the penetration test.

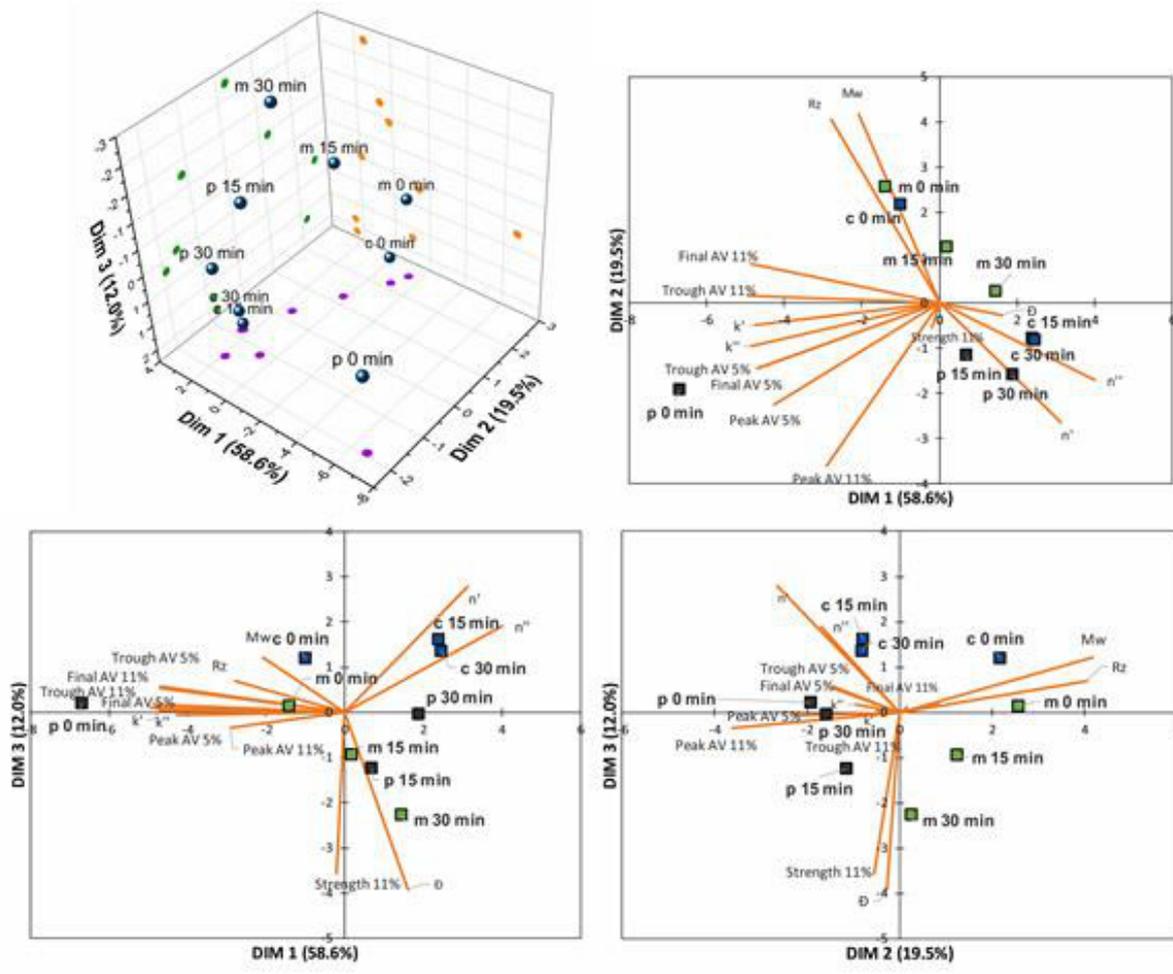


Figure 9. Principal Component Analysis (PCA) which associates native and processed starches with their physical properties. 3D plot represents the observation chart considering the three components (dimensions) with higher explained variance (spheres represent the samples and circles represent the projection of the sample in each 2D plan). 2D plots represent the projection of dimension 1, 2 and 3 combination (squares represent the samples and lines represent the vector correlation among the starch properties). p = potato, c = cassava, m = maize/corn, and the time (min) represent the ozonation time.



Figure S1. Corn starch gels (at 10.7% and 5%) just after gelatinization using the RVA Standard 2 protocol.

APPENDIX D: Properties and possible applications of ozone-modified potato starch

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Properties and possible applications of ozone-modified potato starch

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ABSTRACT

In this work, different properties of potato starch modified by ozone oxidation were evaluated and described aiming to represent different possibilities of industrial application. The most promising results were observed regarding the pasting properties and the gel texture of the starch samples ozonated for 15 and 30 min. These samples presented a higher apparent viscosity and a higher gel strength when gelatinized at 65 and 70 °C, if compared to the native sample. Furthermore, the 15 and 30-min samples retained more water at mild temperatures (~60 °C) than the other samples. These results could be related to the less compact structure of the oxidized starches after the ozone processing due to the cleavage of their glycosidic bonds and the presence of electronegative groups. Not only do these characteristics facilitate the water absorption and gelatinization of the samples at mild temperatures, they also favour the granular disruption at higher temperatures (above 85 °C). The data reported in this work broadens the understanding of the ozone-modification process, as well as suggesting possibilities of industrial applications using ozonated potato starch.

1. Introduction

Native starches have specific and limited properties. Consequently, they do not meet all industrial demands. As a result, starch modification became essential.

Starch modification by chemical methods is widely used in many food and non-food applications (Masina et al., 2016). However, the consequent waste generation and high costs demand new alternatives for most of these processes (Liu, Weber, Currie, & Yada, 2003). The ozone is an alternative to current oxidative methods as it is considered a simple technique with environmental claim (Mahapatra, Muthukumarappan, & Julson, 2005).

Starch modification through ozonation is highly relevant from both academic and industrial points of view. Applications of ozone for starch modification have been reported by different authors using different sources, such as rice, maize, potatoes, yams and cassavas (An & King, 2009; Çatal & İbanoğlu, 2012, 2013, 2014; Hui T. Chan, Bhat, & Karim, 2009; Hui Tin Chan et al., 2011; Hui Tin Chan, Fazilah, Bhat, Leh, & Karim, 2012; Klein et al., 2014; Oladebeye, Oshodi, Amoo, & Karim, 2013).

All the results reported in the literature reinforce the effectiveness of the ozone as a potential oxidizing agent in the modification of starches. However, little progress has been made considering the technological

properties of ozonated starches regarding their characteristics under different conditions (different temperature or different starch/water concentrations, in relation to standard of analyses).

In a previous study (Castanha, Matta Junior, & Augusto, 2017), the effect of the ozone on the structure and properties of potato starch was evaluated. The main results are the replacement of the hydroxyl groups by carbonyl and carboxyl groups, as well as to the cleavage of the glycosidic bonds of both amylose and amylopectin molecules (decreasing their sizes), mainly in the amorphous regions of the granules. Considering the properties, significant differences were observed in the starch pasting properties, gel texture and paste clarity, proving the efficacy of the ozone processing. However, to the best of our knowledge, further properties of the ozonated potato starch, such as its pasting properties at different temperatures and starch concentration, as well as the gel texture and the water holding capacity in these conditions, have not been evaluated in the literature. Consequently, this is the objective of this work.

There are several reasons that explain the importance of evaluating modified starch properties under different conditions, instead of using standard protocols of analysis: I. mostly starchy systems undergo mechanical and thermal processes before being used, because in native conditions they are insoluble in water at room temperature and less digested by the human body (Malumba, Doran, Danthine, Blecker, &

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Béra, 2018); II. to achieve the required characteristics for different applications, starch thermal processing is performed under different conditions, which vary widely among industries and among products; III. the behaviour of the starch samples is influenced by factors such as the rate of increase in temperature, starch concentration and shear forces (Anuntagool, Alvarez, & Flick, 2017), making it difficult to predict and control; IV. the starch modification processes can change the granule size, morphology and composition, significantly affecting its gelatinization behaviour (Jane et al., 1999).

Therefore, the standard conditions may not be representative for all industrial applications and evaluation under different conditions may be important to understand possible applications of the modified starches. For example, in a Rapid Visco Analyser (RVA) analysis, the starch concentration is usually fixed at ~10%, although it widely varies in the industry. This variation in the analysis conditions is rarely conducted in the literature.

Considering this scenario, the aim of this paper was to evaluate and describe the properties of potato starch modified by ozone oxidation under different analyses conditions (at different temperatures and starch concentration). In addition, this study aimed to broaden the understanding concerning the impact of this type of modification on the potato starch properties and applications by correlating these properties with the previously reported structural changes undergone by them.

2. Material and methods

2.1. Material

Potato starch was extracted from native potatoes (*Solanum tuberosum* L., Monalisa cultivar) and the proximal composition of both potato tubers and potato starch was described in our previous study (Castanha et al., 2017) as the same batch was used. The main characteristics of the potato starch were: ~28% of apparent amylose content, ~800 mg·kg⁻¹ of phosphorus content and ~99.5% of purity.

All the chemicals were of analytical grade.

2.2. Starch modification

First of all, 700 mL of the starch suspension in distilled water (10% m/m in dry basis) was ozonated for 15, 30, 45 and 60 min, under constant stirring, with a gas flow of 0.5 L·min⁻¹ and an ozone concentration in the gas current of 47 mg O₃·L⁻¹. The ozone was produced in an ozone generator unit (Ozone & Life, O&G Model RM 3.0, São José dos Campos, Brazil) by the corona discharge method from industrial oxygen (95% purity). The glass reactor was cylindrical, with a capacity of 2 L, 56 cm high and 6 cm in internal diameter. The gas was dispersed in the sample through a glass tube, ending in a porous gas disperser to create small bubbles aiming to increase the ozone diffusion in the suspension. After processing, the starch samples were allowed to settle, the upper water was poured, and the samples were dried in an air circulation oven at 35 °C until ~12% of moisture content.

Further details of the ozonation process can be found in our previous paper (Castanha et al., 2017).

2.3. Starch evaluation

2.3.1. Granule properties: particle size distribution (PSD) and zeta potential (ζ)

The PSD of the starch samples was determined using a Laser Analyser (Partica LA-950V2 Laser Particle Size Analyser, HORIBA, Japan) and the data were evaluated using the software LA-950 for Windows (HORIBA, Japan). The samples were dispersed in ethanol (99.5%). The volume-based mean diameter (D[4,3], Eq. 1) and the area-based mean diameter (D[3,2], Eq. 2) were evaluated, where n_i was the number of particles with diameter d_i ,

$$D[4,3] = \frac{\sum_i n_i d_i^4}{\sum_i n_i d_i^3} \quad (1)$$

$$D[3,2] = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \quad (2)$$

The ζ of the starch granules was measured using Zetasizer equipment (Zetasizer Nano ZS, Malvern Instruments, United Kingdom). The starch suspensions were prepared in Milli-Q water (0.01% m/m) and the pH was adjusted 24 h before the analysis to 3.0, 4.0, 5.0, 5.5, 6.5 and 7.0 using 0.1 M HCl or 0.1 M NaOH.

2.3.2. Water absorption index (WAI) and Water solubility index (WSI)

The WAI and the WSI were evaluated as described by Anderson, Conway, and Peplinski (1970), with modifications. 0.5 g of starch (dry basis) was mixed with 6 mL of distilled water in pre-weighed centrifuge tubes. The tubes were then put in the water bath and stirred for 30 min at different temperatures (30, 55, 60, 65, 70, 85 and 95 °C). After heating, the tubes were centrifuged at 3000g for 10 min. After centrifugation, 2 phases were obtained: the supernatant phase, which contains the starch soluble fraction in water; and the precipitated phase, containing the starch insoluble fraction and the water retained by it.

The soluble starch fraction (SS) of the supernatant was determined after drying at 105 °C, to calculate the WSI (Eq. 3). Therefore, the WSI represents the soluble portion (%) of the starch at the given temperature. The precipitated (PT) sample retained in the tube was also weighed. The WAI is an indicator of the starch swelling power and represents the amount of water that 1 g of insoluble starch (IS) was able to absorb and retain (Eq. 4). For both equations: MS is the mass of the starch sample, in dry basis.

$$WSI (\%) = \frac{SS}{MS} \cdot 100 \quad (3)$$

$$WAI \left(\frac{\text{g water}}{\text{g IS}} \right) = \frac{PT - (MS - SS)}{(MS - SS)} \quad (4)$$

2.3.3. Pasting properties

The pasting properties were determined using a Rapid Visco Analyser (RVA-4, Newport Scientific Pvt. Ltd., Australia) with the Thermocline for Windows software (version 3.0). 1 g of starch (correct to 14% moisture basis) was suspended in 27 g of distilled water and analysed (starch concentration ~3.6%). It is important to highlight that the water/sample proportion was different from that conventionally used as the pasting properties of the starch under standard conditions of analysis have already been explored in previous studies (Castanha et al., 2017; Çatal & İbanoğlu, 2012). Furthermore, the standard concentration used in the RVA assays is higher than the regular concentration of industrial applications, especially for food products. Therefore, the RVA analysis was used as a tool to compare the behaviour of the samples under different conditions, and not as a standard analysis to characterize the modified starches.

The suspensions were first held at 40 °C for 1 min, then heated to different maximum temperatures (55, 60, 65, 70, 85 and 95 °C) for 7 min and 30 s. They were then kept at the maximum temperature for 5 min, followed by cooling to 40 °C for 7 min and 30 s, and finally held at 40 °C for 2 min. The maximum temperatures were based on the gelatinization temperature measured using Differential Scanning Calorimeter equipment (DSC-60, Shimadzu, Japan). For the DSC analysis, 3 mg of dry starch was placed in an aluminium pan with 7 μ L of deionized water. The samples in the hermetically sealed pans were equilibrated at room temperature for 1 h before the measurements were taken. The scanning temperature was from 30 to 100 °C and the heating rate was 10 °C·min⁻¹. An empty pan was used as a reference. The DSC results can be found in the Supplementary material (Fig. S1, Table S1).

It is important to highlight that the DSC indicates the temperature at which the starch granules begin to irreversibly change, under quiescent conditions. Therefore, the DSC analysis was performed under standard conditions in order to characterize the gelatinization temperatures of the samples. Moreover, the results were used as a reference to determine the temperature profiles of the RVA analysis.

2.3.4. Optical microscopy

The starch granule surface and swelling characteristics were observed using a light microscope (model L1000, Bioval, Brazil) with a 20 W halogen lamp. 1 mL of the starch dispersion/gel obtained after each Rapid Visco Analyser (RVA) assay was dispersed in 1 mL of distilled water with 50 μ L of Lugol. A drop of this material was placed on a glass slide and covered by a glass cover slip. The magnification used was 100 \times and a microscope digital camera of 1.3 megapixels was used to obtain the images after verifying being representative.

2.3.5. Gel strength

The strength of the potato starch gels was determined by a puncture assay using a Texture Analyser (TA.XT Plus, Stable Micro Systems Ltd., United Kingdom) with a load cell of 50 kgf (490.3 N). The gel obtained in the Rapid Visco Analyser (RVA) (after the analysis was performed at 65, 70, 85 and 95 °C) was stored in a 40 \times 20 mm (diameter \times height) plastic cup for 7 days in a refrigerator (5 \pm 2 °C) and was retained inside a desiccator with water to ensure uniform moisture of the samples. A 0.5 cm cylindrical probe (P/0.5R) was used to penetrate the samples until a distance of 6 mm at 1 mm \cdot s⁻¹. To ensure a constant temperature during the analysis, the samples were removed from the refrigerator and analysed immediately. The force measured by the equipment as a function of the penetration depth was then used to evaluate the sample strength. The total energy required for penetration was then calculated through the area below the “force versus penetration” curve.

2.3.6. In vitro digestibility

The *in vitro* digestibility of the starch samples was determined according to the method described by Englyst, Kingman, and Cummings (1992), considering the modifications proposed by Zhang, Mei, Chen, and Chen (2017). The enzymatic solution used was composed by α -amylase and amyloglucosidase at a ratio of 120 U/mL:80 U/mL.

After preparing them (according to the aforementioned methods), the samples were incubated at 37 °C under stirring (170 RPM), thus simulating their digestion. Then, 5 mL of each sample was collected at different time intervals (20 and 120 min), mixed with 4 mL of ethanol solution (80%) to inactivate the enzymes and analysed using two different methods: glucose content and reducing sugar content.

The glucose content was measured by the glucose reagent kit based on the GOD/POD reaction method (Ref. number 133, Glucose liquiform, Brazil). The reducing sugar content was determined by the Somogyi-Nelson colorimetric method (Nelson, 1944; Somogyi, 1945).

Following the Englyst et al. (1992) terminology, the percentages of glucose and reducing sugar found in each digestion phase were converted to Rapidly Digestible Starch (RDS - Eq. 5), Slowly Digestible Starch (SDS - Eq. 6) and Resistant Starch (RS - Eq. 7) values. G20 and G120 correspond, respectively, to the glucose/reducing sugar contents after 20 and 120 min of digestion, TS is the Total Starch content, and FG is the Free Glucose (glucose content of the sample before the analysis).

$$\text{RDS (\%)} = \frac{(G20 - FG)}{TS} \cdot 100 \cdot 0.9 \quad (5)$$

$$\text{SDS (\%)} = \frac{(G120 - G20)}{TS} \cdot 100 \cdot 0.9 \quad (6)$$

$$\text{RS (\%)} = \frac{(TS - \text{RDS} - \text{SDS})}{TS} \cdot 100 \quad (7)$$

2.4. Experimental design and statistical analysis

Each ozone process was performed four times. The analyses were repeated at least two times for each sample. The average values were calculated and, when appropriate, the Tukey's multiple comparisons were adopted using Statistica 13.0 (StatSoft, USA) software. A significance level of 5% was considered.

3. Results and discussion

In general, our first paper (Castanha et al., 2017) demonstrated that the ozonation process changed the potato starch structure by increasing the contents of carbonyl, carboxyl and reducing sugar (increased $\sim 2.5 \times$ after 60 min) and by decreasing the apparent amylose content (from 28.4 to 21.0 after 60 min), the pH value (from 6.4 to 4.2 after 60 min) and the molecular size (proved by gel permeation chromatography). The crystallinity of the starch granules was not changed. Analysing all the results concerning the starch structure, a mechanism of action of the ozone in the starch was proposed. Furthermore, the morphology of the granules was changed as the ozonated samples presented cracks and fissures. All these changes affected some of the starch properties, as pasting properties (evaluated under a standard protocol of analysis), gel texture and paste clarity. Further properties are now evaluated, focusing on possible applications.

3.1. Properties of the potato starch granule: particle size distribution (PSD) and zeta potential (ζ)

Fig. 1 presents the PSD and the ζ of the native and ozonated potato starches.

The native sample presented a higher D[4,3] (Fig. 1A) if compared to the ozonated samples, while the D[3,2] values were very similar for all the samples (Fig. 1B). The volume-based mean particle diameter (D [4,3]) is more influenced by large particles, while the area-based (D [3,2]) is more influenced by smaller ones (Bengtsson & Tornberg, 2011; Lopez-Sanchez et al., 2011). Therefore, the results indicate that the ozonation process led to a decrease in the diameter of the larger starch granules, having less influence on the smaller ones. This behaviour may be due to the greater accessibility of the ozone to the larger granules during the processing. On the other hand, the ozonated samples presented similar mean particle sizes among them (Fig. 1C).

The ζ (Fig. 1D) of the starch granules was measured and characterized at different pH conditions. At pH 3.0, the samples were beginning to present negative values of ζ . In all the other pH values, however, the ζ of the samples was negative, including in the pH 5.5, presented by the native potato starch sample with no NaOH or HCl addition. The phosphate groups naturally found in the potato starch samples contributed to this behaviour as they are negatively charged (Szymońska, Molenda, & Wieczorek, 2015). It is important to note that, even within the same sample, the variation of the ζ values was high. This was probably due to the potato starch granular size, which is too large to accurately determine the ζ values.

The combination of both particle size and ζ gives an indication of the stabilization capacity of emulsions and suspensions, as well as the particle aggregation. However, the changes in the PSD and in the ζ are more significant in the case of starch nanoparticles (size < 100 nm, according to Jiang, Mashayekhi, and Xing (2009)), which are much smaller than the granules analysed in this paper. In addition, the observed changes on the potato starch granule PSD and ζ were small in the conditions applied in the present work.

3.2. Pasting properties

The RVA was selected to evaluate the properties of the starch samples under non-standard protocols as this analysis measures the temperature at which the swelling of the starch granules starts to affect

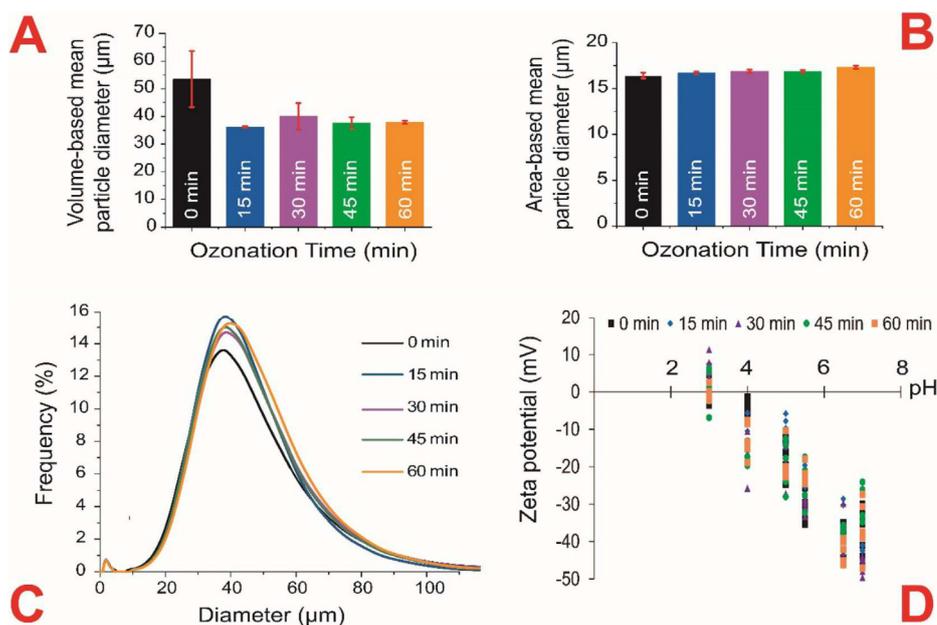


Fig. 1. (A) Volume-based mean particle diameter (D[4,3]) of the native and ozonated potato starches. (B) Area-based mean particle diameter (D[3,2]) of the native and ozonated potato starches. (C) Effect of the ozonation time (0–60 min) on the particle size distribution of the potato starch (based on volume). (D) Zeta potential of the starch granules: all points measured in the analysis are shown in the graphic. Vertical red bars represent the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the apparent viscosity of the system, under heating and shear (Liu, 2005; Yu & Christie, 2001), thus better reproducing some industrial conditions. Therefore, the RVA analysis was performed using an initial and final temperature of 40 °C, and maximum temperatures ranging from 55 °C (lower than the Onset temperature of gelatinization for all samples, according to the DSC analysis – Table S1, Supplementary Material) to 95 °C (higher than the Endset temperatures measured for all the samples, according to the DSC analysis – Table S1, Supplementary material). The RVA curves are shown in Fig. 2.

The first interesting point that can be observed in Fig. 2 is the shape of the native starch curves, which is different from the commonly observed curves for potato starch. This shape is due to the water/sample proportion used in this study (3.6%, instead of the standard 10.7%). By changing the proportion, the shape of the native starch curve was completely different from the “standard” curve, without the well-known peak apparent viscosity and breakdown regions. This behaviour was probably due to the lower interaction between the swelled granules and the continuous phase, as usually occurs at low concentrated solutions (Biliaderis, 2009; Booth & Bason, 2007).

By analysing the sample's behaviour at different temperatures (Fig. 2), it can be observed that no increase in the apparent viscosity was observed at 55 and 60 °C for all the samples.

However, the most remarkable results were observed at 65 and 70 °C. At these temperatures, the native starch presented only a small increase in its apparent viscosity, while the 15-min ozonated sample presented the highest apparent viscosity among all the treatments. At 70 °C, the apparent viscosity of the 15-min treatment was the highest obtained by this sample (even higher than at 85 or 90 °C). It suggests that the ozonation process, at mild conditions, can be a good alternative to obtain starches that form viscous pastes at moderate temperatures (65–70 °C).

On the other hand, the native starch sample started to present an apparent viscosity higher than that of the 15-min treatment at 85 °C reaching an even higher value at the end of the 95 °C analysis. In contrast, at higher temperatures, the ozonated samples presented lower

apparent viscosity values if compared with their results at 70 °C.

3.2.1. Microscopic evaluation of the starch pastes

The starch samples were collected at the end of each RVA analysis and evaluated using a light microscope (Fig. 3). This helped to explain this phenomenon, by visually demonstrating the changes observed in the RVA results. A comprehensive scheme (Fig. 4) representing the general steps of the starch gelatinization is also available and is a reference to analyse the microscopic images, thus determining at which stage of the gelatinization process the samples are. The scheme was based on the definitions found in studies by Atwell, Hood, Lineback, Varriano-Marston, and Zobel (1988), Liu (2005) and Xie et al. (2007).

After the RVA analysis at 55 °C, where no apparent viscosity variation was observed, no particular changes in the granule morphology were observed (Fig. 3), indicating that the majority of the granule population did not absorb enough energy to swell at this temperature.

At the RVA analysis at 60 °C, no apparent viscosity variation was observed either (Fig. 2), however some of the starch granules swelled slightly in all the starch samples (Fig. 3). This can be related to the fact that at lower temperatures, there is less swelling and less leaching of starch molecules into the continuous phase and, consequently, lower interactions among the granules. Thus, despite the fact that some of the granules are irreversibly changed, the apparent viscosity of the system was not measurable by the equipment.

At this point, it is important to mention that each particular granule has a specific behaviour and may not perform in the same way the majority granule population does under the same conditions (Huang et al., 2017; Lund & Lorenz, 1984; Tao et al., 2018). The DSC and the RVA results provide a general view of the granular population, while the granules can be observed through the microscope individually. Therefore, this result demonstrates that the ozonation process can change the distribution of gelatinization behaviour among the granules, even though it does not change the average behaviour.

After the RVA analysis at 65 °C, the native starch granules were swollen and their granular integrity (shape and surface) were

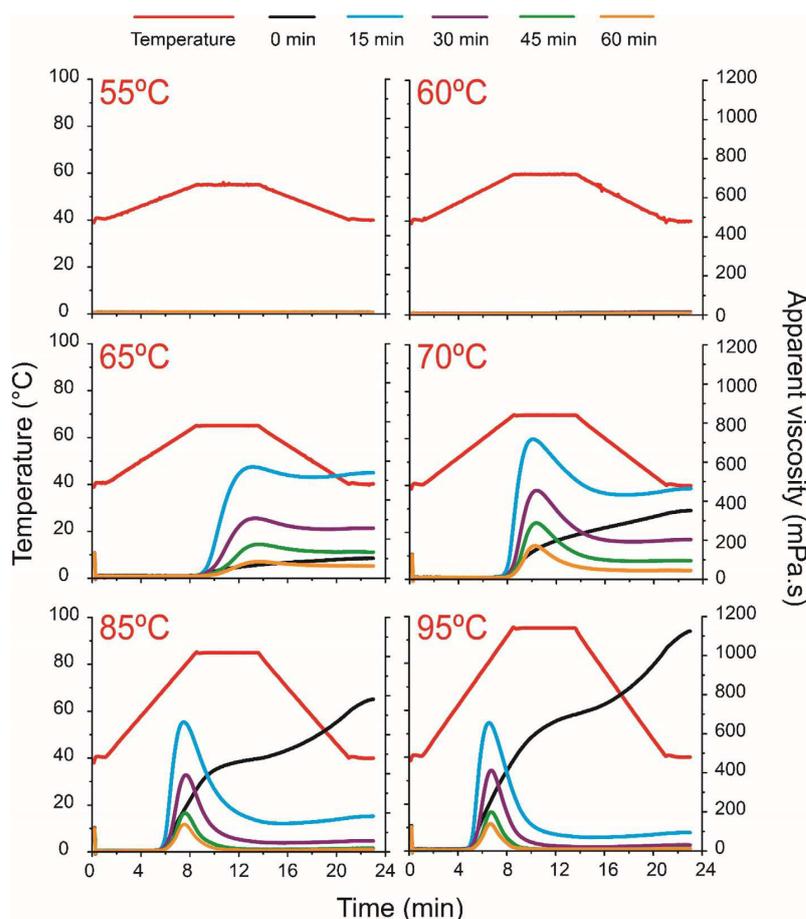


Fig. 2. Rapid Visco Analyser (RVA) curves of the native and ozone-oxidized potato starches analysed under different maximum temperatures.

maintained (Fig. 3). On the other hand, the 15 and 30-min samples also presented whole granules, but their surface was not so marked. In addition, their granules were apparently bigger in size than the native ones, which could explain their higher apparent viscosity. The 45 and 60-min samples also presented highly swollen granules, but their granular surfaces were less distinguishable, as if they were disassembled, indicating that the ozonation at more severe conditions make the starch granules more “fragile”.

At 70 °C, all samples presented gelatinized granules (Fig. 3). However, the higher the ozonation time, the less distinguishable the surfaces of the ozonated samples. This indicates that their granular structure no longer withstand these analysis conditions (temperature and shear). Apparently, the largest granules are those of the 15-min sample, which can explain the higher apparent viscosity peak reached by this sample at these conditions (Fig. 2).

In the RVA analyses at 85 and 95 °C, the native starch sample began to exhibit a higher apparent viscosity than the oxidized ones (Fig. 2). By analysing their granules, it is possible to see (Fig. 3) that all the samples started to present granular rupture and higher amylose leaching with increasing temperatures. At 85 °C, the native and the 15-min samples presented distinguishable dispersed granules, while the 30-min and, especially, the 45 and 60-min ozonated samples only presented fragments of the granules dispersed within the system. Similar behaviour occurred after the 95 °C analysis, except that, in this case, the samples of 0 and 15 min were more degraded, while the more ozonated samples

were completely disintegrated.

3.2.2. Discussion

Our theory to explain this behaviour is related with the starch structure, which undergoes considerable changes after the ozonation process. Considering the native starch, whose granules carry intact molecules (*i.e.*, non-oxidized), higher temperatures are required to disrupt the bonds of the crystalline region of the granules and lead to a granular swelling, until reaching the point of changing the apparent viscosity of the system. On the other hand, in the oxidized samples, the hydroxyl groups were partially replaced by carbonyl and carboxyl groups, which are electronegative and increased with increasing ozonation time (Castanha et al., 2017). These groups can cause an electrostatic and steric repulsion, resulting in bulkier molecules. Moreover, the cleavage of the glycosidic bonds of the ozonated starch molecules resulted in smaller molecules (Castanha et al., 2017). Both factors probably contributed to the crystalline double helix weakening, thus promoting the flow of water into the granule of the less ozonated samples (15 and 30 min) at lower temperatures if compared to the native sample.

It is important to notice, however, that the same factors that cooperate with the water uptake and granular swelling in the less oxidized samples were responsible for impairing the granule swelling of the highest oxidized samples (45 and 60 min). This occurs since they were developed in an intensified way: there are less hydroxyl groups

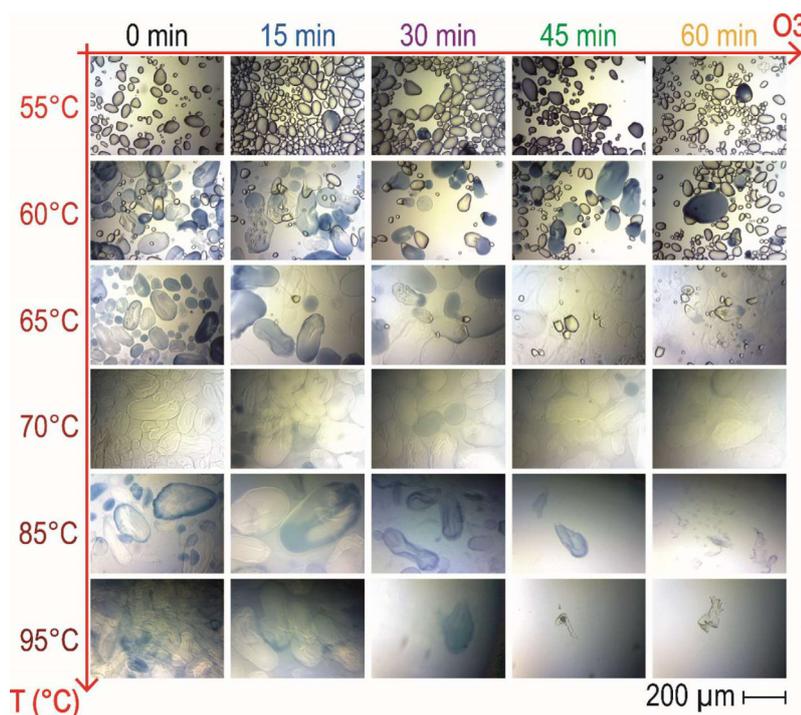


Fig. 3. Light microscopy images of the native and the ozonated potato starch evaluated after the Rapid Visco Analyser (RVA) assays. The black line in the lower right corner measures 200 μm. Lugol solution was used to better visualize the starch granules.

available to form hydrogen bonds with the water molecules and, as these granules are “damaged” (due to the molecular breakdown), they are less capable of maintaining their integrity under stirring and heating.

To sum up, the RVA results (Fig. 2) showed that, in general, the native starch demanded higher temperatures (above 85 °C) to present a more significant apparent viscosity variation compared to the 15 and 30-min ozonated samples. On the other hand, the 15 and 30-min samples presented higher apparent viscosity results at milder temperatures of gelatinization (65 and 70 °C), if compared to the native sample in the same conditions. The 45 and 60-min samples were too degraded to present a good performance in the RVA assays, showing a negligible apparent viscosity in all evaluated conditions. Consequently, focusing on possible industrial applications, it is necessary to understand the capacity of these samples to interact with water (absorption and solubility), as well as the properties of the obtained gel and the

starch digestibility.

3.3. Water absorption index (WAI) and water solubility index (WSI)

The WSI and WAI of the starch samples are presented in Fig. 5

In general, considering the increase in both ozonation time and temperature (Fig. 5A), the solubility of the starch fractions in water (WSI) increases. Considering only the effect of the temperature on the WSI, the results show that the solubility of the ozonated samples rises more significantly with increasing temperatures above 70 °C, when compared to the variation at mild temperatures. This behaviour is due to the starch gelatinization, in which one of the consequences is leaching of the amylose molecules (which are soluble). Besides, as the amylopectin molecules are the main component of the granules, their solubilization (due to the weakening of the crystalline part during the gelatinization phenomenon) also influences these results. On the other

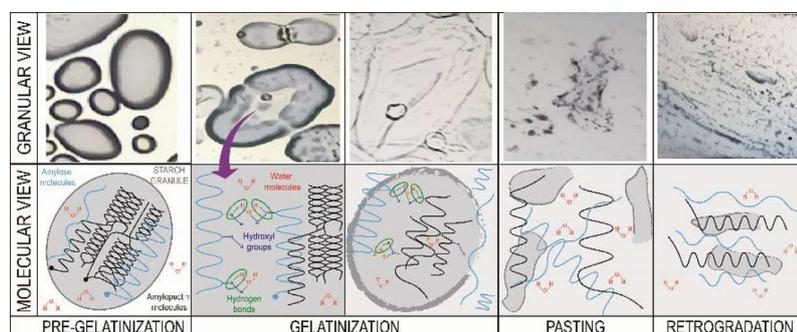


Fig. 4. Graphic representation of the starch pre-gelatinization, gelatinization, pasting and retrogradation phenomena. Top line: granular view (actual images from potato starch). Bottom line: molecular view (representation). Based on the theories presented in the works of Atwell et al. (1988), Liu (2005) and Xie et al. (2007).

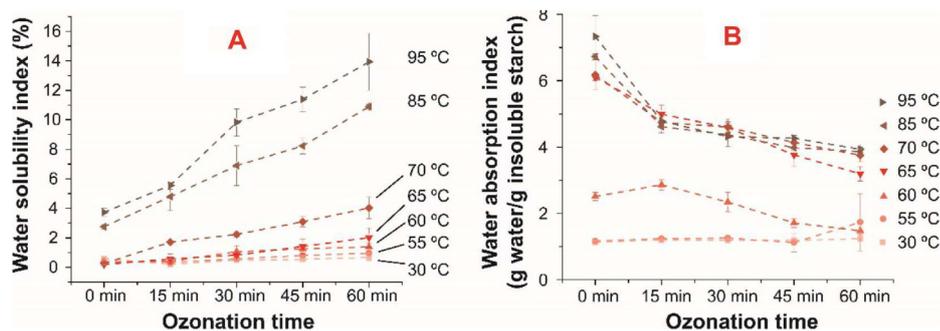


Fig. 5. (A) Water Solubility Index and (B) Water Absorption Index of the native and ozonated samples at different temperatures (30, 55, 60, 65, 70, 85 and 95 °C). Vertical bars represent the standard deviation. The curves are traced to facilitate interpretation.

hand, for the native (0 min) sample, at 70 °C, the WSI value was similar to those observed at lower temperatures, and were only significant above 80 °C. As discussed previously (for RVA analysis), this behaviour is probably due to the fact that the native starch granules are intact: the glycosidic bonds of the molecules are undamaged and their hydroxyl groups were not replaced by electronegative groups, bonding strongly to each other. Thus, their molecules are more tightly bonded, which hinders their solubility in water if compared to the ozonated starches.

Similarly, considering only the effect of the ozonation time on the WSI, it can be observed that the longer the ozonation time, the higher the granule solubility (Fig. 5A). These results are in accordance with the previous discussions and results: the ozonation led to a molecular cleavage and to an increase in electronegative groups (Castanha et al., 2017), and were both responsible for easily disrupting the starch granules, especially at higher temperatures. Thus, the longer the ozonation time, the higher the molecular breakdown and, therefore, the higher the solubility of the granules.

On the whole, the WAI rose when the temperature was increased, but decreased with increasing ozonation time (Fig. 5B). It is important to remember that the WAI represents the amount of water that the starch can absorb and retain, being a property of high importance in the food industry. Considering the effect of the temperature on the WAI, it is clear that the higher the temperature, the higher the water absorption. As in the WSI, the most significant increase occurred at temperatures above 70 °C.

On the other hand, the effect of the ozonation time on the WAI behaviour was more complex to understand. In general, it followed the same pattern observed in the RVA analysis: the 15-min sample presented a slightly higher water absorption capacity at 60 °C. This indicates that the structural changes shown by this sample after the ozonation process allowed the uptake and retention of water in the granules, without using elevated temperatures. Considering the increasing temperatures, however, the native (0 min) starch granules started to be able to absorb and retain more water than the ozonated samples, probably due to their non-damaged structure, which was capable of retaining more water at higher temperatures. Oladebeye et al. (2013) also observed a decrease in the WAI with increasing ozonation time in starches from white and red cocoyam, attributing the results to structural disintegration.

Therefore, the WAI results suggest that the ozonation process can be an alternative to obtain starches with good properties of water absorption and retention if the gelatinization process is conducted at moderate temperatures (above 60 °C). At higher processing temperatures, however, the absorption capacity of the ozonated starches are lower than that of native starch. On the other hand, they presented a higher solubility, which can be interesting for products such as chocolate powder or instant soups, which require easy solubilization.

3.4. Gel strength

The samples obtained after the RVA analysis were stored for 7 days under refrigeration (5 ± 2 °C) before the analysis. After this period, their strength was evaluated through instrumental texture (penetration test). The results obtained are shown in Fig. 6. The samples obtained after the RVA analysis at 55 and 60 °C were not evaluated since they could not form gel, presenting a separation of phases.

The 45 and 60-min ozonated samples did not form consistent gels after RVA analysis (Fig. 6), probably due to the fact that their molecules were too small and with many electronegative groups to permit a strong molecular association in these analysis conditions.

Similar gel characteristics (weak gels) were observed for the native (0 min) starch samples after the RVA analysis at mild temperatures (65 and 70 °C), but for another reason: as explained in previous discussions, the mild temperatures were not sufficient to induce a complete gelatinization of the granules. Therefore, the molecules were not able to re-associate and form firm gels after cooling as they were still in a granular structure. In fact, the strength of the native starch gels gradually grew with increasing processing temperature, achieving its maximum value in the gel obtained after the RVA analysis at 95 °C (0.86 mJ).

On the other hand, the 15-min ozonated sample showed opposing behaviour: a maximum strength was reached at 65 °C, with decreasing gel strength and increasing processing temperature. Molecules with intermediate chain lengths are more likely to reassociate and bind than larger molecules, explaining the gel strength of this sample. As the temperature increased, however, this sample (which was more fragile than the control sample) was more degraded and did not form a consistent gel.

Interestingly, the 15-min sample reached a gel strength value at 65 °C (0.82 mJ) and 70 °C (0.80 mJ) that was similar to the gel strength presented by the native sample at 95 °C (0.86 mJ). This result is highly relevant because the 15-min ozonated starch sample can be used in products where the strength of the gel after storage is more interesting than the pasting properties of the starch, with energy gain (since less heat is needed to paste this starch). This is the case, for example, of meat products, where both water retention and texture are common demands for starch application.

Considering the 30-min ozonated sample, its higher gel strength value was obtained at 65 °C (0.41 mJ), and was half of the force value reached by the 15-min sample in the same conditions. When the processing temperature was increased, the 30-min sample, similarly to the 15-min sample, presented weaker gels.

To visually demonstrate the effect of ozonation on the potato starch gels, a video illustrating the flow of the starch samples gelatinized at 70 °C after 24 h and after 7 days of refrigerated storage was made, and is available at: <https://www.youtube.com/watch?v=WtYxreS1-78>.

In fact, the results obtained in the texture analysis are consistent with those observed in the other analyses. In the native sample, mild

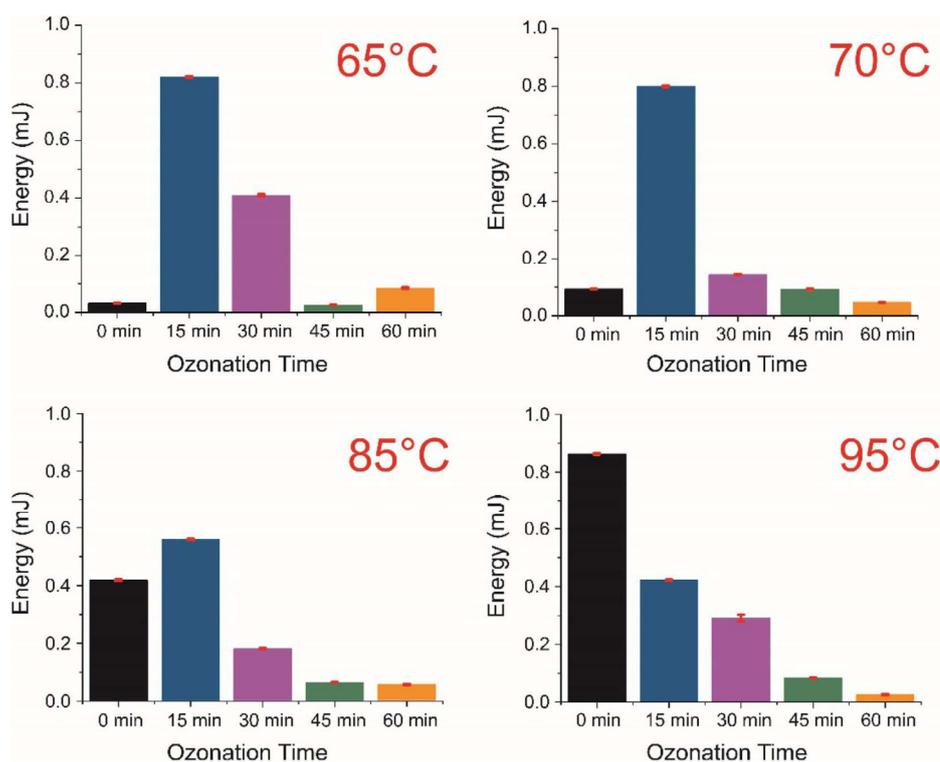


Fig. 6. Strength of the potato starch gels (required energy for penetration of the gels in a puncture test) with different ozonation processes. The gels were obtained after RVA assays at different temperatures (65, 70, 85 and 95 °C). Vertical red bars represent the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

temperatures were not sufficient to permit a gel formation, but the gel strength increased when the temperature rose. On the other hand, in the 15-min sample, the mild temperatures were sufficient to allow the gelatinization of the starch and consequent gel formation after cooling. The 30-min sample presented similar results, but with lower intensity of gel strength. And the highly-ozonated samples (45 and 60-min) did not form consistent gels (they also did not present viscous pastes in the RVA analysis – Fig. 2) under any condition.

3.5. *In vitro* digestibility

The RDS fraction of the potato starch samples is shown in Fig. 7. The remaining value is basically composed by the SDS fraction, whereas the RS fraction was practically negligible for the ozonated samples, presenting a small percentage ($5.3 \pm 2.4\%$, through the glucose analysis approach) in the native sample.

Fig. 7 shows that concerning the reducing sugar content, there is no statistical difference ($p < .05$) between the native and the ozonated samples up to 45 min. Furthermore, no significant difference was observed between the ozonated samples. Only the native and the 60-min sample can be considered different and the reducing sugar content of the 60-min sample is higher than the native sample. In fact, virtually all the 60-min sample was converted into reducing sugar after 20 min of digestion.

Considering the glucose method, the RDS content of the native potato starch sample was lower if compared to the ozonated samples (~73%, while the ozonated samples presented values above 84%). On the other hand, the 15, 30 and 45-min samples can be considered significant equals ($p > .05$) regarding their glucose content, as well as the 30, 45 and 60-min samples.

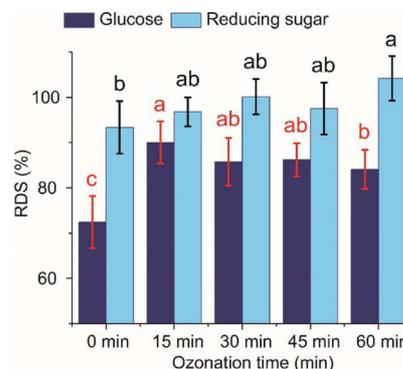


Fig. 7. Rapidly digestible starch (RDS) content of the potato starch samples after *in vitro* digestibility analysis based on both glucose and reducing sugar contents. Vertical red and black bars represent the standard deviation; different small letters indicate significant differences ($p < .05$) among the samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Since the reducing sugar content analysis is based on the evaluation of reducing terminals of the molecules, this analysis is able to detect both the oligosaccharide and glucose molecules. The glucose content analysis, on the other hand, only detects glucose molecules. Considering this information, the RDS result presented in the Fig. 7 is coherent, since the RDS observed through the glucose content was lower than that observed using the reducing sugar content.

In the literature, the RDS fraction of gelatinized potato starch varied

from ~73% (Villas-Boas & Franco, 2016) to ~85% (Dupuis, Tsao, Yada, & Liu, 2017), considering studies using methodologies similar to those used in this paper (glucose value after 20 min of digestion), showing a similarity with the reported results (Fig. 7).

The digestibility of the potato starch samples observed in Fig. 7 are also in accordance with the other results presented in this work. The water easily permeates the ozonated starches, which presented lower chain-length molecules. Therefore, it is reasonable to assume that their molecules are more susceptible to an enzymatic degradation. Furthermore, the electronegative nature of carbonyl and carboxyl groups can somehow affect the interaction of the molecules with the enzyme, thus affecting the starch digestibility.

These results may not be considered desirable if considering the trend for products with higher levels of resistant starches as they are continuously related to health benefits for the consumers (Asp & Björck, 1992; Magallanes-Cruz, Flores-Silva, & Bello-Perez, 2017). However, the ozonation can be considered as a good method to increase the enzymatic susceptibility of the starches and can be used for other industrial purposes (as pointed out in the “Technological applications” section of this study). Furthermore, should be mentioned that this result only evaluated the gelatinized starch (which was boiled in water for 20 min). Different results are expected if gelatinization is conducted at mild temperatures, leading to different applications. It is worth mentioning that more studies are needed to evaluate this subject.

Even so, it is important to highlight that we used the methodology proposed by Englyst et al. (1992), which classifies the starch in three fractions of digestibility rates: rapidly digestible starches (RDS), slowly digestible starches (SDS) and resistant starches (RD). Although it is a good approach to evaluate the digestion tendency of isolated starches (without being included in a food matrix, as in this paper), this method cannot be considered a reliable reproduction of the human organism. Thus, a digestibility curve based on the enzymes kinetics would be a better way to evaluate the samples.

4. Final remarks

Considering all the properties presented by the ozonated starch samples, some suggestions for future applications can be made. Regarding the pasting properties, which indicated that the ozonation process can be a good alternative to obtain starches that can form viscous pastes at moderate temperatures, a direct application would be in some conventional products, such as instant soups.

The WAI results suggested that the ozonation process can be a good alternative to obtain starches with good properties of water absorption and retention by conducting the gelatinization process at moderate temperatures. This is the case of many industrial products, especially food, such as meat products for example.

Additionally, in both cases, the ozonation process can be an alternative to obtain hydrocolloids for nursing-care foods, especially dysphagia foods (Funami, 2011) as the gelatinization must be formed at small/moderate temperatures and since syneresis must be avoided due to the risk of aspiration. It is worth mentioning that more studies are needed to evaluate this subject.

Considering the high enzymatic susceptibility presented by the ozonated samples, starch modification using the ozone technology could be applied as pre-treatment for enzymatic processes.

5. Conclusions

The results reported in this paper open up the possibilities of applications for the modified potato starch using the ozone technology. For instance, our results demonstrated that potato starch submitted to mild ozone processing can retain more water after gelatinization at mild temperatures than the native one, resulting in higher apparent viscosity and gel strength. Besides, the *in vitro* digestibility indicated that the ozonated starches presented a higher enzymatic susceptibility

if compared to the native sample. On the other hand, the higher ozonated samples were able to completely gelatinize at small temperatures and were also more soluble if compared to the native and less ozonated samples.

All these observed properties can be useful for different technological applications, especially regarding processes/products that demand specific characteristics, such as viscous pastes or higher water retention at moderate temperatures (between 60 and 70 °C).

Consequently, ozone processing can be an alternative to obtain starches with new and potentially useful properties for different industrial applications, with the extra advantage of being obtained through a safe and environmentally friendly technology (ozone).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2018.09.064>.

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Declarations of interest

None

Contributors

N. Castanha designed and performed the experiments, evaluated the data and wrote the manuscript. D.N. Santos performed the *in vitro* digestibility experiments and also evaluated and discussed the data. R.L. Cunha evaluated the data and the manuscript. P.E.D. Augusto designed the experiments, evaluated the data, wrote the manuscript and directed and managed the team. All the authors discussed and approved the manuscript.

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APPENDIX E: Combining ozone and ultrasound technologies to modify maize starch

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Combining ozone and ultrasound technologies to modify maize starch

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ABSTRACT

In this work, maize starch was modified using ultrasound (US) and ozone (O₃) treatments, each one alone and also in combination. The starch molecular structure, granule characteristics and properties were evaluated. The US treatment alone did not show influence on the starch physical characteristics. On the other hand, the O₃ treatment, alone or in combination with US, led to significant changes on starch molecules by increasing carbonyl and carboxyl groups and the apparent amylose content, while decreasing pH and the starch molecular size distribution. The granules' particle size distribution (PSD), their morphology and crystallinity were not affected by any of the treatments. Regarding the starch properties, water absorption index (WAI), water solubility index (WSI), pasting properties and gel strength were clearly more affected by the ozone treatment as compared with the ultrasound treatment. However, the paste clarity was significant higher when the combined treatments were applied, especially when US was used before O₃. These results are prompting the hypothesis that the US treatment improved the subsequent action of O₃.

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1. Introduction

Starches are the main energy reserve of plants and are abundantly found in nature. Obtained from renewable sources, being inexpensive and very versatile material in terms of functional properties, starches are used in several industries, such as food, paper, textile and pharmaceutical [1,2]. The modification of starches is a highly industrially employed technique, since it is an applicable form to improve the functional properties of native starches [3,4] — which are limited by nature. However, nowadays as important as to obtain starches with better properties, is using efficient, safe and environmentally friendly methods to achieve it.

Among the starch modification methods, the most industrially employed are the oxidative ones [3]. However, these processes are usually performed using chemical agents, and are being questioned regarding their impacts on consumers and the environment, which has led to a search by new technologies [3,5]. Among the possible alternatives to chemical methods, ozone (O₃) has been shown to be one of the most promising, as it is highly oxidative [6] and has the advantage of being an environmentally friendly technology, since it quickly decomposes to oxygen [7]. Ozone has already been studied as an oxidizing agent for the modification of a wide range of starch sources, such as potato [8,9], rice [10] and cassava [11]. Starch modification caused by this

treatment has been attributed to the generation of electronegative groups and the hydrolysis of the glycosidic bonds of the starch molecule, thus reducing the starch molecular size [8,10,12,13]. However, it is important to emphasize that the botanical source and, consequently, the structure of the starch, has a great influence on the ozone action, and further studies in this field are still required.

On the other hand, physical modification methods, among which we highlight US, can be used to produce modified starches with specific functional properties, having as a positive aspect the fact that no chemical agent is used in the process [3]. High intensity ultrasound treatment is able to change the material properties (i.e. physical disruption, acceleration of some chemical reactions), due to a formation of intense cavitation [14,15]. The modification of starch technological properties using ultrasound has already been studied and proposed for different starch sources, such as rice [16], oat [17], cassava [18], and maize [19,20]. US is claimed to cause starch modifications such as disintegration of granules [21–23], and molecular size reduction [21,24]. However, structural modifications of biopolymers by US treatment are reported to be highly dependent on the US conditions and the material source [25,26]. In fact, some of the results presented in the literature are contradictory. These differences in the observed results emphasize that the lack of standardization during these treatments does not enable to establish definite conclusions about the treatment efficacy and its effects on the starch molecular structure. Moreover, some works in the literature do not follow good practices in ultrasound processing, which can compromise the obtained results [27].

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Finally, the combination of ozone and ultrasound treatments have been little explored. Only two published works explore this subject [28,29]. However, both papers report only rheological and thermal properties of the modified starches, but do not provide information on structural changes neither potential correlations between the modified starch structure and its properties. Consequently, the mechanisms of the combined action of ultrasound and ozone on starch, remains unexplored.

In order to meet the industrial demand for starches with specific characteristics, considering safe and environmental needs, the objective of this work aims to evaluate the structure and the properties of modified starches using combined ultrasound and ozone treatments. Due to its high industrial importance, maize (corn) starch was used as the targeted starch.

2. Material and methods

2.1. Starch sample and processing conditions

Native maize (corn) starch (~28% of amylose, Argo CS 3400) was provided by “Ingredion Brasil Ingredientes Ltda”.

The ozone (O_3) treatment used was similar to the one reported in previous work [8], which is schematically illustrated in Fig. 1. In short, 700 mL of starch suspension (10% m/m, wet basis) in distilled water was placed in a glass reactor (capacity: 2 L, height: 56 cm, internal diameter: 6 cm) and processed for 15 min at 25 °C. The ozone was generated from industrial oxygen (95% purity; constant flow at 1 L/min) using an ozone generator unit (Ozone & Life, O&G Model RM 3.0, São Jose dos Campos, Brazil). The gas ($O_2 + O_3$) stream, with an ozone concentration

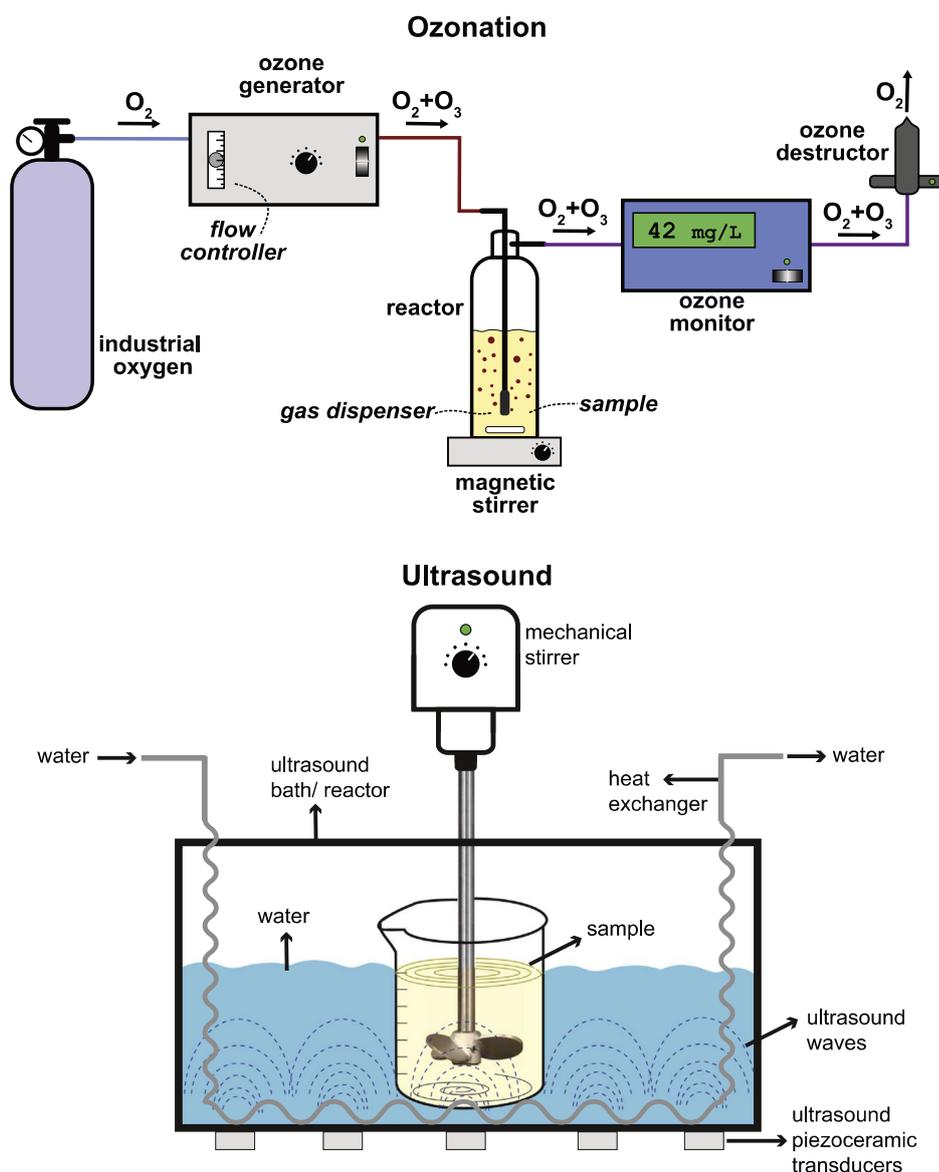


Fig. 1. General aspect of the ozonation (up) and ultrasound (down) systems.

of 42 mg O₃/L of mixture, was introduced in the reactor, being bubbled in the starch suspension. After processing, the water was separated from the sample, and an air circulation oven (at 35 °C) was used to dry the starch (until ~12% of moisture content). The samples were sieved (250 µm) before the analyses.

To perform the physical modification using ultrasound (US), a starch suspension in distilled water (700 mL, 10% w/w) was prepared inside a glass beaker and processed in an ultrasonic bath (Q13/25, Ultronique, Brazil). The US conditions were: processing time of 8 h, temperature of 24–26 °C, frequency of 25 kHz and volumetric power of 72 W/L — which was calculated by the calorimetric method, according to the described by O'Donnell et al. [30]. The temperature was controlled using a heat exchanger inside the US bath (the recirculating water was provided by an external water bath). The ideal location of the samples inside the US bath was evaluated according to the good practices described in the literature [27], ensuring homogeneity and the exposure of the sample to the highest US intensity. The bath was filled with 6 L of distilled water mixed with 15 drops (~0.5 g) of dishwashing detergent (lemon dishwasher, Ypê, Brazil), to decrease the surface tension of the solvent. To prevent decantation of the starch granules during the treatment, a mechanical stirrer was used and located as illustrated in Fig. 1. After processing, the water was separated from the sample, and an air circulation oven (at 35 °C) was used to dry the starch (until ~12% of moisture content). The samples were sieved (250 µm) before the analyses.

Maize starch was treated by US or O₃ individually. Furthermore, the combination of these two treatments was performed as follows: immediately after ozone treatment, the starch sample was transferred to a beaker and treated by ultrasound, using the same conditions previously described. Moreover, the order of the combined treatment changes. That is, another sample, after being processed by ultrasound, was transferred to the reactor and ozonated. The ozone processing (indicated as O₃) was combined with the ultrasound processing (indicated as US), is distinguished as US-O₃ or O₃-US according to the order of the treatments. In summary four different treatments (O₃, US, US-O₃ and O₃-US) were used to assess the physical modifications achieved by these treatments.

2.2. Starch molecular evaluation

2.2.1. Carbonyl and carboxyl groups

The methodology described by Chattopadhyay, Singhai and Kulkarni [31], with modifications, was used to evaluate the carboxyl content. In details, ~3 g of starch was suspended in 25 mL of 0.1 M HCl and stirred for 30 min. The samples were then filtered (205 µm) and washed with distilled water. The retained samples were mixed with 300 mL of distilled water and gelatinized in a boiling water bath for 15 min. The sample, still hot, was titrated with NaOH (0.1 M) until pH 8.3, using an automatic titrator (Plus Titrino 848, Metrohm, Herisau, Switzerland). A non-modified sample (control) was regarded as the blank. Using Eq. (1), the “carboxyl groups per 100 glucose units (COOH/100GU)” was calculated. In the equation: V_s = volume of NaOH used for the sample, V_b = volume of NaOH used for the blank, M = molarity of NaOH, and S = the mass of sample (dry basis).

$$\frac{\text{COOH}}{100\text{GU}} = \frac{(V_s - V_b) \cdot M \cdot 0.045 \cdot 100}{S} \quad (1)$$

The methodology described by Smith [32], with modifications, was used to evaluate the carbonyl content. In details, ~4 g of starch was suspended in 100 mL of distilled water and completely gelatinized in a boiling water bath for 30 min. The gels were then cooled (to ~40 °C) and their pH was adjusted to 3.2 using HCl 0.1 M. The samples were then mixed with 15 mL of hydroxylamine solution (10 g of hydroxylamine hydrochloride + 40 mL of 0.5 M NaOH solution + distilled

water until reach 200 mL) and kept in a water bath at 40 °C for 4 h. The sample was then titrated with HCl (0.1 M) until pH 3.2, using an automatic titrator (Plus Titrino 848, Metrohm, Herisau, Switzerland). A mix of all the reagents, without any starch sample, was regarded as the blank. Using Eq. (2), the “carbonyl groups per 100 glucose units (CO/100GU)” was calculated. In the equation: V_b = the volume of HCl used to test the blank, V_s = the volume of HCl required in the titration, M = molarity of HCl, and S = the mass of the sample (dry basis).

$$\frac{\text{CO}}{100\text{GU}} = \frac{(V_b - V_s) \cdot M \cdot 0.028 \cdot 100}{S} \quad (2)$$

2.2.2. pH

The pH was determined as specified in Adolfo Lutz Institute [33], using a starch dispersion (10%) and a calibrated potentiometer (Tecnal, TEC-5 mode).

2.2.3. Molecular size distribution

A gel permeation chromatography was used to evaluate the molecular size distribution of the samples. A CL-2B gel (Sigma, Sweden) was packed in a GE XK 26/70 column (diameter: 2.6 cm, height: 70 cm). The samples' preparation was performed as follows: 0.1 g of starch was mixed with 10 mL dimethylsulfoxide (DMSO, 90%) and heated in boiling water bath for 1 h. The obtained gels were slowly stirred overnight at room temperature (~25 °C). After this process, an aliquot of 3 mL of sample (containing ~30 mg of starch) was mixed with 10 mL of ethanol (99.5%), which promoted the starch precipitation. To isolate the precipitated starch, the samples were centrifuged at 1210 RPM for 30 min. Before injecting the samples in the column, the precipitated starch was mixed with 9 mL of hot distilled water kept in a boiling water bath until complete dissolution, according to Song and Jane [34]. 4 mL of the obtained solution (starch concentration of ~3 mg/mL) was then upwardly eluted in the column, using a solution of 25 mmol/L of NaCl + 1 mmol/L of NaOH as mobile phase, at a rate of 60 mL/h. A fraction collector (Gilson model FC203B, Middleton, England) was used to collect 4 mL of samples/ tube, and the content of each tube was analysed using the blue value method [35], at 630 nm wavelength, in a spectrophotometer (Femto, Model 600S, São Paulo, Brazil).

2.2.4. Apparent amylose content

The ISO methodology [36] was used to analyse the apparent amylose content of the samples. In short, the starch sample was mixed with ethanol and sodium hydroxide and gelatinized in a boiling water bath for 10 min. After the gelatinization, the obtained sample was mixed with NaOH 0.09 M, and an aliquot of this new solution was then mixed with acetic acid (1 M), iodine solution (10% KI and 5% I₂ in distilled water) and distilled water (until reach 100 mL). The intensity of the obtained blue colour (starch iodine complex) was estimated by measuring the samples' absorbance at 620 nm using a spectrophotometer (Femto, Model 600S, São Paulo, Brazil). A standard curve of amylose/amylopectin previously prepared was used as reference.

2.3. Starch granules evaluation

2.3.1. Granule morphology

Images of the starch granules, showing their morphology and the possible changes in the starch surface after processing, were obtained by scanning electronic microscopy using a FEI Inspect F50, (Japan) operating at an acceleration voltage of 2.0 kV and available at LNNano, Campinas, Brazil.

To prepare the samples, a double sticky carbon tape was fixed on a circular aluminium stub, and dry starch was sprinkled on the tape. A brush was used to ensure a uniform layer of powder particles. The stubs were then coated with gold (~4 nm) and then evaluated in the microscope using an Everhart-Thornley Detector (ETD) for secondary electrons.

2.3.2. Granule relative crystallinity

The crystallinity patterns of the starch granules were evaluated using an X-ray diffractometer (Rigaku Miniflex 600 RXD, Tokyo, Japan), using copper radiation and the following test conditions: scan rate of 2°/min at an angle 2θ ranging from 3 to 50°, with 40 kV and 15 mA. To ensure a constant moisture of the samples before the assays, the starch samples were kept for 10 days in a desiccator containing saturated BaCl₂ solution (25 °C, a_w = 0.9) at the bottom. The relative crystallinity (RC) of the starch granules was calculated, as described by Nara and Komiya [37], using the Origin software version 9.1 (Microcal Inc., Northampton, MA, USA). The 2θ angle selected for the calculation ranged from 3 to 36°

2.3.3. Granules size distribution

The particle size distribution (PSD) of the starch granules was measured using a Laser Analyser equipment (Partica LA-950V2 Laser Particle Size Analyser, HORIBA, Japan). To avoid increase in the granules' size due to water uptake, the samples were dispersed in ethanol (99.5%). The obtained data were evaluated using the software LA-950 for Windows (HORIBA, Japan). The area-based mean diameters (D [2,3]) calculated by Eq. (3) were evaluated, where n_i was the number of particles with diameter d_i.

$$D[3,2] = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \quad (3)$$

2.4. Starch properties evaluation

2.4.1. Pasting properties

The pasting properties were determined using the RVA-4 equipment (Newport Scientific Pvt. Ltd., Australia, with the Thermocline for Windows software, version 3.0). The Standard 2 program was selected (held at 50 °C for 1 min, heating to 95 °C at 6 °C/min, held at 95 °C for 5 min, cooled to 50 °C at 6 °C/min, and held at 50 °C for 2 min). In each cycle, 3 g of starch sample (14% moisture basis) and 25 g of distilled water were used.

2.4.2. Gel texture

The gel strength was determined using a Texture Analyser (TA.XT Plus, Stable Micro Systems Ltd., Surrey, UK) with a load cell of 50 kgf (~490 N). The samples obtained after the RVA assays were stored in 30 × 20 mm (diameter × height) plastic cups for 24 h at 5 ± 2 °C. To ensure uniform moisture of the samples, they were held in a desiccator with water at the bottom. After storage, the samples were carefully removed from the cups, smeared with mineral oil, and uniaxially compressed until 75% of its height using a plate probe (P/100) with 100 mm of diameter. The stress was calculated as the ratio of the force measured by the equipment and the samples' transversal area. The relative strain was calculated as the ratio of the maximum strain measured by the equipment and the samples' height.

2.4.3. Paste clarity

The method described by Craig et al. [38], with the modifications proposed by Aplevicz and Demiate [39], was used to measure the paste clarity of the samples. This method is based on the transmittance measurement (T%) of the starch pastes, which were obtained after the gelatinization of 0.2 g of starch in 20 mL of distilled water, using a boiling water bath for 30 min. To ensure homogeneity of the samples, each tube was individually stirred 5 times every 5 min. Before the assays, the tubes were cooled to ambient temperature (~25 °C). The pastes' absorbance was measured at 650 nm using a spectrophotometer (Femto, Model 600S, São Paulo, Brazil).

2.4.4. Water absorption index (WAI) and water solubility index (WSI)

The method described by Anderson et al. [40], with modifications, was used to evaluate the WAI and the WSI of the starch samples. In details, 0.5 g of starch (dry basis) was mixed with 6 mL of distilled water in pre-weighed centrifuge tubes. The samples were then heated using different temperatures (60, 70, 80 and 90 °C) for 30 min in a water bath with slow stirring. After heating, the tubes were centrifuged (1210 RPM for 10 min), and both supernatant and precipitated were collected. The supernatant phase (containing the soluble starch) was dried at 105 °C in pre-weighed glass plates and the soluble fraction (SF) was determined, being used to calculate the WSI (Eq. (4)). The precipitated fraction (PF) retained in the tube (containing the starch insoluble fraction and the water retained by it) was also weighed to calculate WAI (Eq. (5)). For both equations: M is the mass of the starch sample (dry basis).

$$WSI (\%) = \frac{SF}{M} \cdot 100 \quad (4)$$

$$WAI \left(\frac{\text{g water}}{\text{g IS}} \right) = \frac{PF - (M - SF)}{(M - SF)} \quad (5)$$

2.5. Experimental design and statistics

Each process (O₃, US, O₃-US and US-O₃) was performed in triplicate and the analyses were performed at least in duplicate for each sample. The averages and the standard deviations were calculated and, when appropriate, the Tukey's multiple comparisons were used. A significance level of 5% was considered. A non-modified maize starch sample (indicated as "control") was used for comparisons.

3. Results

3.1. Starch molecular structure

Results of the starch molecular structure evaluated using carbonyl and carboxyl groups content, pH, apparent amylose content and molecular size distribution are shown in Fig. 2.

3.1.1. Molecular oxidation: Carbonyl, carboxyl and pH

Considering the carbonyl content (Fig. 2A), the US sample did not present a significant difference ($p < 0.05$) compared to the control sample, while the O₃, O₃-US and US-O₃ samples had an increased content of carbonyl groups compared to the control and the US treated sample. Concerning carboxyl groups (Fig. 2A), the O₃ and the US-O₃ samples had a significant increase ($p > 0.05$) when compared to the control, while the US and the O₃-US samples did not. The pH (Fig. 2B) of the samples decreased after the modification, except for the US sample, which did not show a significant variation when compared to the control.

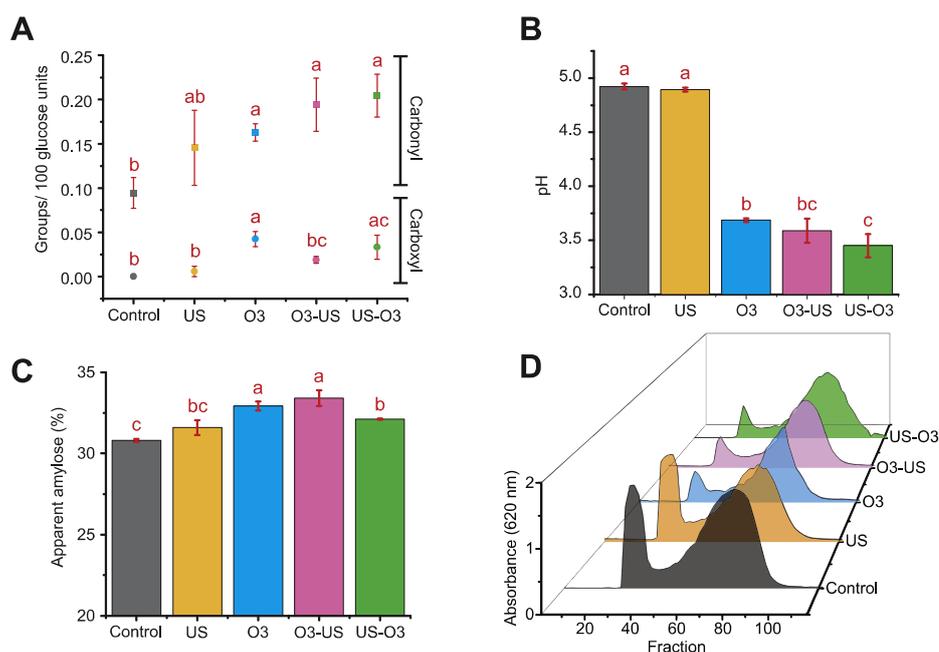


Fig. 2. Carbonyl and carboxyl groups (A); pH (B); apparent amylose content (C); and molecular size distribution profile (blue value method) (D) of the control and modified (US, O₃, O₃-US and US-O₃) maize starch samples. Vertical red bars represent the standard deviations. Results labelled with the same small letter do not differ significantly ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In fact, the carbonyl and carboxyl groups are expected to increase after oxidative processes, as a consequence of the oxidation of the hydroxyl groups [41,42]. Also, as those groups increase, the pH of the samples tends to decrease, due to the formation of carboxylic acids, which is in accordance with results obtained in this work. Therefore, the modification involving the use of ozone (alone or combined with US), in general, led to an increase of the carbonyl and carboxyl groups (Fig. 2A) and a decrease of pH (Fig. 2B). The only exception was the O₃-US sample, whose carboxyl content was not statistically different from the control sample, despite presenting a higher value than the control. One possible explanation for this behavior can be that the combination of US and O₃ led to a higher formation of carbonyl groups, rather than carboxyl groups, as can be observed in the Fig. 2A. Even so we highlight the differences are small, and they can also be related with sample/process variability. On the other hand, US processing alone, as expected, did not affect the hydroxyl groups of the starch molecules.

3.1.2. Molecular depolymerization: apparent amylose content and size exclusion chromatography

Proceeding with the molecular evaluation, another change that usually occurs with modified starches is the hydrolysis of their glycosidic bonds, leading to a depolymerization of their molecules (both amylose and amylopectin), resulting in shorter molecular chains [8,41,43,44]. The blue value method, based on the starch iodine complex, is one of the most useful methods to evaluate the changes in this degree of polymerization [45,46]. Therefore, the modified samples were evaluated using two different approaches, both based on the blue value method: the apparent amylose content and size exclusion chromatography.

The apparent amylose content (Fig. 2C) increased after the modification processes. The exception, once again, was the US treatment, which did not show a significant change ($p < 0.05$) when compared to the control. As previously mentioned, the apparent amylose analysis is, in fact, an indirect evaluation of the iodine affinity of the starch molecules,

which can increase or decrease depending on its molecular size. Therefore, the ozone process partially depolymerized the starch molecules, whose residues showed higher iodine affinity, when compared to the US and control samples. In fact, the most notable differences on the molecular size distribution profile (Fig. 2D) were observed on samples that were treated with ozone (O₃, O₃-US and US-O₃).

The apparent amylose content variation after an ozonation process is contradictory in the literature. Some works reported increased values after the modification processes [12], while other report decreases [8]. Since this analysis is based on the iodine affinity of the amylose and amylopectin molecules, it is possible to assume that the contradictory results may be related to the molecular chain-length and electronegative groups obtained after the modification process. Castanha et al. [8], for example, studied the modification of potato starch, which is well known to have amylopectin molecules with long chains and high iodine affinity [47], in addition of having a high level of phosphate groups. All those characteristics may have influenced the measured apparent amylose decrease, since the molecules may have become less prone to bind iodine after the modification. An opposite behavior was observed by Oladebeye et al. [12], indicating that ozone, as in our case, improved the iodine affinity of the molecules — more than simple affirm “ozone increased the amylose content”. Summarizing, this analysis seems to be dependent of the starch source, and consequently of the molecular size distribution and electronegative groups, and processing conditions. Consequently, it appears the apparent amylose content assay may not be a conclusive response of the effects of the ozone modifications. To overcome that weakness in the analysis, gel permeation chromatography was used to help the understanding of starch molecular changes upon the modification processes.

Chromatography results (Fig. 2D) showed a notable difference between the ozonated samples (O₃, O₃-US and US-O₃) when compared to the US and control samples, with a clear decreasing of the first peak. Gel permeation chromatography analysis divides the starch molecules based on their sizes and ramifications: long and branched

molecules elute first, forming the first peak (usually associated with amylopectin molecules), while shorter and less branched molecules elute later, forming the second peak (usually associated with amylose molecules) [48]. Therefore, the decrease of the first peak after the ozone treatment indicates that the molecules with longer chain-lengths (probably mostly amylopectin) are depolymerized by this process.

3.1.3. Molecular structure evaluation: overview

Considering all the molecular results (Fig. 2), it is possible to observe some patterns. In general, the effect of combining the technologies (US + O₃) did not present a significant difference if compared to the O₃ results or among themselves. It indicates that ozone processing, at the conditions applied in this work, had a higher influence on the starch

molecular modification when compared to the ultrasound processing. Furthermore, the order at which the processes were combined (US-O₃ or O₃-US) apparently had no influence on starch molecular changes.

It is important to notice, however, that despite of no statistical definitive, the US-O₃ treated sample showed a tendency to have a higher carbonyl content (Fig. 2A) and a lower pH (Fig. 2B), when compared to the other samples. This sample showed a significant decrease in the apparent amylose content (Fig. 2C) when compared to the O₃ and O₃-US samples, and its molecular-size distribution showed a second peak eluting later, when compared to the other samples (Fig. 2D), indicating that the molecular size of this sample was smaller when compared to the other ones. All these results, despite apparently not significant, played an important role on the properties of the US-O₃ sample, as discussed in next sections. Furthermore, this information can indicate that the

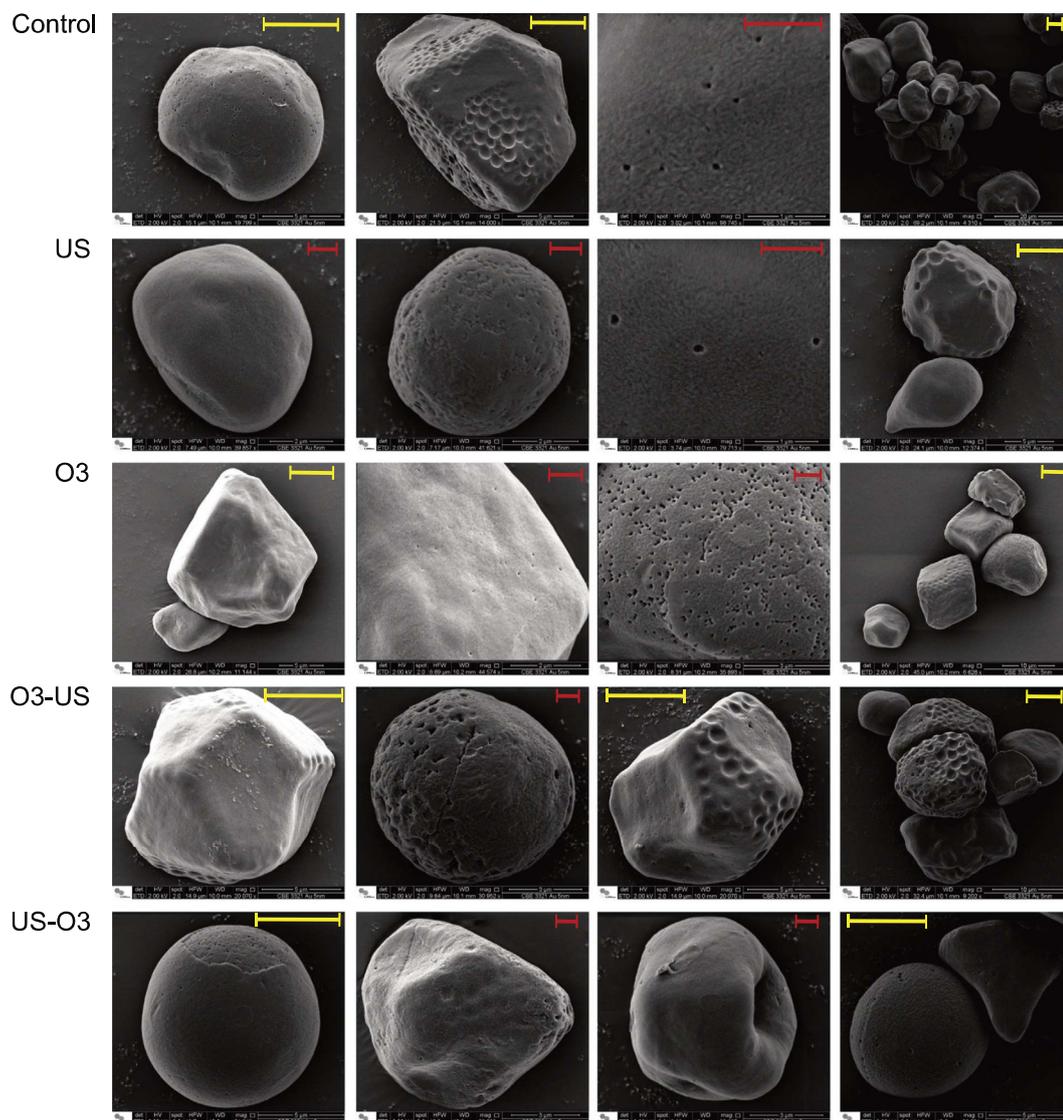


Fig. 3. Scanning electronic microscopy (SEM) images of the control and modified (US, O₃, O₃-US and US-O₃) maize starch samples. Yellow lines measure 5 μm, and red lines measure 1 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

US alone processing does not change the starch molecules, but it can promote changes on the starch granules, that affect the way how ozone processing interacts with it (i.e. US-O₃ treatment).

Summarizing, US process by itself did not led to any significant changes in the starch molecules structure. The carbonyl and carboxyl groups, and consequently the pH, remained unchanged, as well as its molecular size distribution and apparent amylose content. Conversely, ozonated samples (O₃, O₃-US and US-O₃), with few exceptions, showed significant changes ($p > 0.05$) in all the evaluated parameters, indicating that the ozone processing, alone or in combination with the US, led to significant molecular changes in maize starch.

Therefore, it is important to evaluate the effect of these processes on the starch granule, in order to better understand each effect and test our hypothesis concerning the US treatment, as follows.

3.2. Characteristics of the starch granules

The characteristics of the starch granules were also investigated, in order to elucidate if the modification processes had any influence on their morphology. As discussed in the **Material and methods** section, methods used were scanning electronic microscopy (SEM, Fig. 3), particle size distribution (PSD, Fig. 4) and X-ray analysis to measure granule crystallinity (Fig. 5).

3.2.1. Granule morphology: scanning electronic microscopy (SEM)

The starch granule morphology is illustrated in Fig. 3. The control sample presented small and irregular granules, as expected for native maize starch, and some of the granules also presented pores, roughness, craters and depressions on their surfaces. Similarly, the modified samples exhibited irregular granules whose surface had pores, roughness, craters and depressions, meaning that no notable changes were observed between the control and the modified samples.

Other works on the literature reports the appearance of pores on the surface of different starch samples after ultrasound processing [44,49,50]. Some hypothesis can be raised to explain why no differences were observed in the present work. The first one is related with the naturally heterogeneous and irregular granules shown by the native maize starch, which makes it difficult to notice any changes on their characteristics (in special using a qualitative tool such as SEM). Another explanation is that the US and O₃ treatments, with the conditions applied in this work, were not enough to change the maize starch granules' morphology. In fact, Sujka and Jamroz [44], by comparing the US effect on the surface characteristics of different starch samples, observed that potato and wheat starches were more affected by the US treatment than rice and maize starches, which have smaller granules.

It is important to mention, however, that although US and O₃ processing was not sufficient to change the granule morphology, results presented next and related to the starch properties reinforces the hypothesis that US processing promotes some changes on the granules, which further affects how ozone processing interact with them (US-O₃ treatment). Our hypothesis is that those changes are originated at the centre of the granules. Sujka and Jamroz [44], using transmission electronic microscopy (TEM), observed not only pores, but also a cavity of irregular shape in the centre of the granules after ultrasound processing, and those cavities were independent of the starch botanical origin. Further studies are needed to clarify this topic.

3.2.2. Granule size: particle size distribution (PSD)

PSD results of the control and modified starch samples, as well as its mean particle diameters, are illustrated in Fig. 4. As illustrated, PSD of the modified samples did not present any remarkable difference when compared to the control (Fig. 4), regardless of having been processed with ozone and/or ultrasound. Luo et al. [50] did not observe changes on the maize starch shape and particle size after processing with ultrasound. Castanha et al. [9], on the other hand, reported a decrease on the volume-based mean particle diameter of potato starch processed with ozone, indicating that the process decreased the size of the bigger granules, but not of the smaller ones. The authors concluded that the bigger granules could be more accessible to ozone treatment. It is interesting to note that Carmona-Garcia et al. [51] indicates that larger granules are also more affected by the US waves than smaller ones, which can partially explain the results obtained in this work (potato starch granules are much higher than maize).

Results showed that the US and O₃ treatments, did not promote apparent changes on the granules' morphology, neither changed their sizes and shapes.

3.2.3. Granule crystallinity: X-ray diffraction

The crystallinity characteristics of the native and modified starch granules are illustrated in Fig. 5. Firstly, control and modified starches can be classified as samples of an A-type, which is typical of cereals [52,53]. In fact, after the modification processes, no changes were observed on the X-ray diffraction patterns of the samples, which maintained the same pattern and relative crystallinity ($p < 0.05$).

Furthermore, some authors [54,55] have suggested a new approach to evaluate the X-ray diffraction patterns of starches. In this approach, the X-ray patterns are compared to an α -amylose standard (PDF 43-1858), available in a database of Powder Diffraction Files (PDF-4) [56]. It worth to be mentioned that the standard amylopectin pattern is still

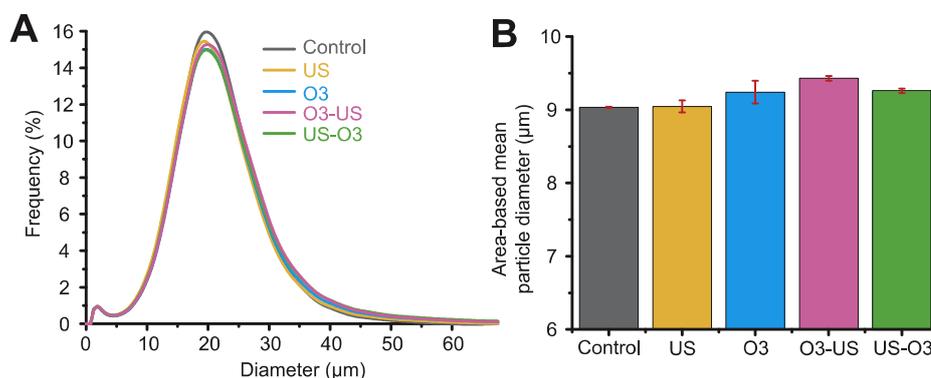


Fig. 4. Particle size distribution (PSD) based on the volume (A); and area-based mean particle diameter: $D [2,3]$ (B) of the control and modified (US, O₃, O₃-US and US-O₃) maize starch samples. Vertical red bars represent the standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

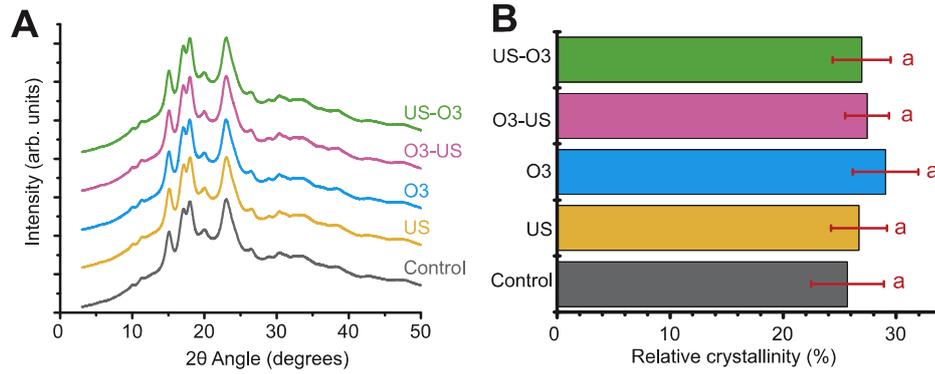


Fig. 5. X-ray diffraction patterns (A); and relative crystallinity (B, calculating from 2θ angle from 3° to 36°) of the control and modified (US, O₃, O₃-US and US-O₃) maize starches. Horizontal red bars represent the standard deviations. Results followed by the same small letter do not differ significantly ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

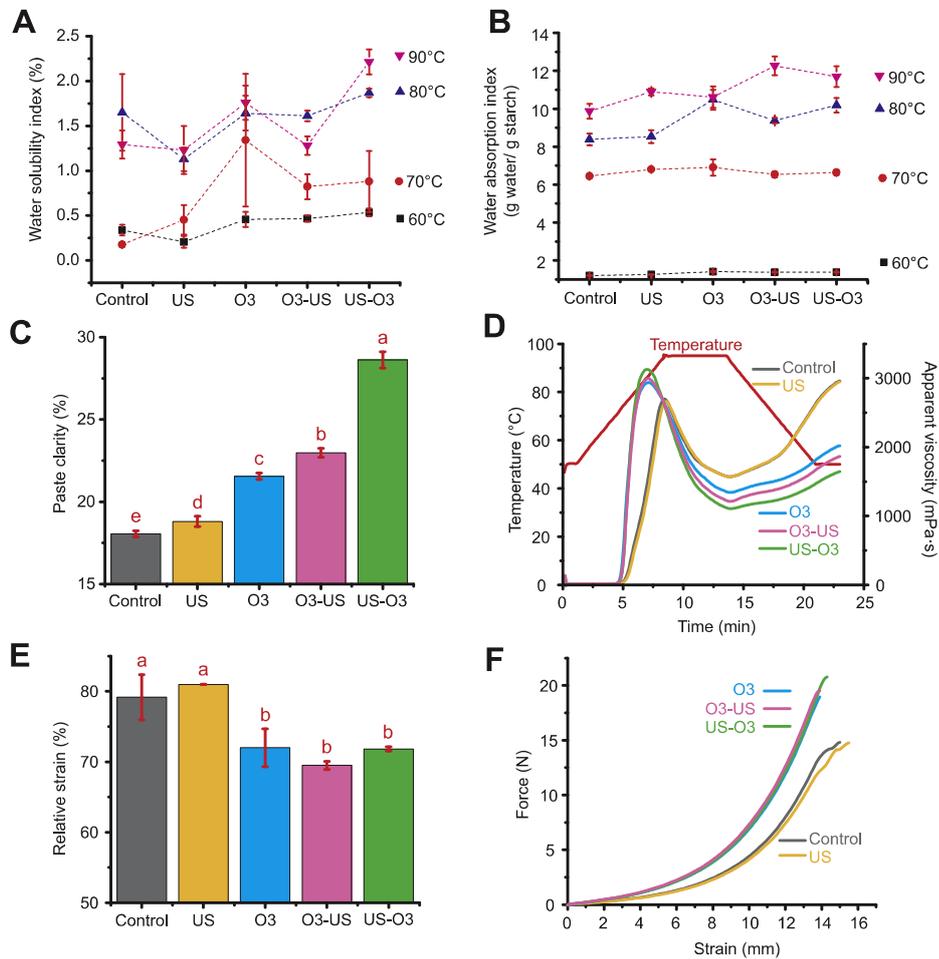


Fig. 6. Water solubility index (WSI) (A); water absorption index (WAI) (B); paste clarity (C); pasting properties (D); relative strain of rupture during compression of the starch gels (E); and stress until rupture of the starch gels (F) of the control and modified (US, O₃, O₃-US and US-O₃) maize starch samples. Vertical red bars represent the standard deviations. Results followed by the same small letter do not differ significantly ($p < 0.05$). Dashed lines on (A) and (B) are traced only to facilitate the interpretation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Water absorption index (WAI) and water solubility index (WSI) of the control and modified (US, O₃, O₃-US and US-O₃) maize starch samples. Average ± standard deviation. Results followed by the same small letter do not differ significantly ($p < 0.05$).

Water absorption index (g water/g starch)																
Samples	60 °C				70 °C				80 °C				90 °C			
Control	1.2	±	0.0	c	6.4	±	0.1	a	8.4	±	0.3	b	9.9	±	0.4	c
US	1.3	±	0.0	b	6.8	±	0.1	a	8.5	±	0.3	bc	10.9	±	0.2	bc
O ₃	1.4	±	0.0	a	6.9	±	0.4	a	10.5	±	0.5	a	10.6	±	0.6	bc
O ₃ -US	1.4	±	0.0	a	6.5	±	0.2	a	9.4	±	0.1	cd	12.3	±	0.5	a
US-O ₃	1.4	±	0.0	a	6.6	±	0.1	a	10.2	±	0.4	ad	11.7	±	0.5	ab

Water solubility index (%)																
Samples	60 °C				70 °C				80 °C				90 °C			
Control	0.3	±	0.1	cb	0.2	±	0.0	b	1.7	±	0.4	ab	1.3	±	0.2	bc
US	0.2	±	0.1	c	0.5	±	0.2	ab	1.1	±	0.1	b	1.2	±	0.3	b
O ₃	0.5	±	0.1	ab	1.3	±	0.7	a	1.6	±	0.2	ab	1.8	±	0.2	ac
O ₃ -US	0.5	±	0.0	ab	0.8	±	0.1	ab	1.6	±	0.1	ab	1.3	±	0.1	bc
US-O ₃	0.5	±	0.0	a	0.9	±	0.3	ab	1.9	±	0.0	a	2.2	±	0.1	a

under study, and that the amylose sample used as reference for the PDF database was not from maize starch. Nevertheless, this approach allows to compare each peak with its corresponding source. For example, the peaks located at $\sim 20^\circ$ and $\sim 22.5^\circ$ are related to the amylose crystalline structure. Therefore, by analysing results using this approach, it is possible to say that neither the amylose nor the amylopectin crystals were affected by the modification process, since the peaks locations were maintained. These results are also supported by the relative crystallinity results (Fig. 5B). Similar results (no changes on the crystalline domains) were found by Luo et al. [50], while studying maize starch modification using ultrasound, and by Castanha et al. [8], while studying potato starch modification using ozone.

3.2.4. Granule structure evaluation: overview

To sum up, regarding the starch granules, it was possible to conclude that their characteristics were apparently maintained, since no changes on their morphology, PSD and crystallinity were observed after the modification processes. However, internal changes were not measured, but as stated by other researchers (e.g. Sujka and Jamroz [44]) using TEM, observed internal modifications could be responsible for observed changes on the starch molecules and their properties after treatments.

3.3. Starch properties

Practical applications of modified starches are one of the main goals of the modification processes. Therefore, some of the functional properties of the modified starches were evaluated and correlated with the structure characteristics described earlier. Water absorption (WAI) and solubility (WSI) indexes, paste clarity, pasting properties (RVA) and gel strength are illustrated in Fig. 6. Tables containing the main

results and statistical analysis concerning the WAI and WSI (Table 1) and RVA (Table 2) analyses are also presented.

3.3.1. Water absorption (WAI) and water solubility (WSI) indexes

As expected, WSI (Fig. 6A) increased with increasing temperature. At 60 °C, the ozonated samples (O₃, O₃-US and US-O₃) showed a tendency to present a higher solubility, although the changes did not result statistically different. At 70 °C, the same behaviour was observed, being the O₃ sample the only that presented a significant difference ($p > 0.05$) when compared to the control sample. At 90 °C, the US-O₃ sample presented the higher solubility of all samples, being statistically equal only to the O₃ sample. Overall, results did not follow a linear increase and it is possible to conclude that O₃ processing apparently increased the starch solubility. On the other hand, US processing alone had no influence on the starch solubility, which is in accordance with results previously discussed (no molecular or granular changes were observed for this sample).

WAI results (Fig. 6B) were also temperature dependent and increased with temperature. The highlights, once again, are observed in the ozonated samples (O₃, O₃-US and US-O₃). At 80 °C, the O₃ and the US-O₃ treatments showed the highest WAI, followed by the O₃-US treatment, whereas at 90 °C, the O₃-US and US-O₃ treatments showed the highest WAI values, being the only treatments different from the control ($p > 0.05$). The US treatment showed no significant differences with the control at temperatures $> 70^\circ\text{C}$. However, at 60 °C, this sample presented a higher absorption capacity when compared to the control sample, indicating that the US treatment may have affected the starch structure (as previously discussed) in a way that promote the increase of the granules water uptake capacity. In fact, as this analysis is temperature-dependent, those results can be related with the integrity

Table 2

Main parameters of the pasting properties of the control and modified (US, O₃, O₃-US and US-O₃) maize starch samples. Average ± standard deviation. Results followed by the same small letter do not differ significantly ($p < 0.05$). AV = apparent viscosity. Relative breakdown was calculated as: (breakdown / peak AV) · 100. Relative setback was calculated as: (setback / trough AV) · 100.

Samples	Peak AV (mPa·s)				Trough AV (mPa·s)				Relative breakdown (%)				Final AV (mPa·s)				Relative setback (%)				Pasting T (°C)			
Control	2703	±	16	c	1571	±	48	a	42	±	2	c	2961	±	28	a	89	±	5	a	76	±	1	a
US	2694	±	2	c	1615	±	18	a	40	±	1	c	2975	±	17	a	84	±	3	a	75	±	3	ab
O ₃	2960	±	1	b	1350	±	47	b	54	±	2	b	2041	±	5	b	51	±	5	b	72	±	1	b
O ₃ -US	3162	±	13	a	1170	±	35	c	63	±	1	a	1721	±	4	c	47	±	5	b	72	±	1	b
US-O ₃	3206	±	23	a	1106	±	17	c	66	±	0	a	1629	±	18	d	47	±	4	b	71	±	3	b

of the granules, since a granule with a damaged structure can present better water absorption at lower temperatures than at higher ones where they may breakdown faster [57].

Sujka et al. [44], processed maize starch in water using an ultrasonic homogenizer, observing an increase of the swelling power and solubility of the samples. Similarly, Luo et al. [50] observed an increase of the swelling power and solubility of normal maize starch processed with ultrasound. Both authors hypothesized (but did not demonstrate) that the results may be related to depolymerization of the starch molecules after ultrasound processing, which was not observed in our work (Fig. 2C and D). It is important to remark that in the mentioned studies the concentration of the processed starch slurries was higher (30%). This and a different system used compared to our work may have influenced the results.

On the other hand, increasing solubility after ozonation were observed by different authors [9,12,13], while the WAI presented different results depending on the starch source and process conditions. Despite different results, the authors related the observed behaviour with the depolymerization of the starch molecules and with the oxidation of the hydroxyl groups to carbonyl and carboxyl groups after the ozone processing, which is in accordance with our results (Fig. 2).

3.3.2. Paste clarity

Paste clarity (Fig. 6C) was significant higher ($p > 0.05$) for all modified starches when compared with the control. The US-O₃ sample stood out, presenting the highest value, which was ~61% higher than the control sample. This is a remarkable result by considering the importance of the paste clarity for several starchy final food products [44].

Our theory to explain this property (US-O₃ sample presenting a higher paste clarity) is once more related to the possible modification caused by the previous US treatment on the internal structure of the maize starch. Although no apparent increase in pores on the surface of the samples was observed, results are showing that the US treatment apparently promoted ozone action, by facilitating its action on the interior of the starch granule. In fact, the weakening of the granules' structure induced by the US treatment has been reported in several works, and it is already being used to facilitate other subsequent processes, such as to facilitate both acid and enzymatic starch hydrolysis and to obtain porous starch granules using amylase, or starch nanoparticles [57,58].

The evidence for this hypothesis is the US-O₃ sample presenting some particularities when compared to the O₃-US sample, like for example the tendency of this sample to have a higher carbonyl content (Fig. 2A) and a lower pH (Fig. 2B), a significant decrease on the apparent amylose content (Fig. 2C), an apparent lower molecular size (Fig. 2D), and higher paste clarity.

3.3.3. Pasting properties and gel texture

The RVA (Fig. 6D) and the gel texture (Fig. 6E and F) presented the same pattern observed throughout this work, in which the ozonated samples (O₃, O₃-US and US-O₃) stood out from the others, agreeing with the previous results. In fact, the US sample did not differ significantly from the control sample in any pasting or gel texture parameters. In general, the ozonated starch samples (O₃, O₃-US and US-O₃) presented higher paste viscosities, lower relative setbacks and harder and less elastic gels (evidenced by the lower relative strain, Fig. 6E). Those characteristics can be useful in some food applications, such as gravies, dips, sauces and puddings [59].

However, the effect of combining the technologies was evidenced on the RVA analysis, indicating that, despite no molecular or granular significant differences between them or when compared to the O₃ sample, their pasting properties had some particularities. Among the results, we highlight the peak apparent viscosity (AV), which was higher when applying the combination of the technologies (US-O₃ and O₃-US), and

their relative setback, which was lower. Those results indicate that the starches were modified by the combined treatments and showed a more consistent paste and a lower tendency to retrogradation. Our results are in discordance with the observed by İbanoğlu et al. [28] while studying the effects of the combination of ozone and ultrasound on the rheological properties of rice starch. The authors observed a higher viscosity after the ultrasound processing, while after the ozone processing and its combination with ultrasound, the starch viscosity decreased. Also, in their work, the order at which the processes were applied had no influence on the results.

3.3.4. Starch properties evaluation: overview

Summarizing, in general the starch properties were affected by the treatments. The ozone process, alone or in combination, once more showed different results when compared to the control and starches treated only with US, which was similar to the control sample. However, results show that the US treatment is apparently able to change the starch structure, improving ozone action. As previously discussed, the main hypothesis for this behavior is possible internal modifications within the granules caused by the ultrasound action. Therefore, although the US technology seemed to be ineffective when using alone, it could be very useful to potentialize the ozone effects, proving to be a potential effective alternative for starch modification.

4. Conclusions

The results illustrated that, at the conditions applied in this work, the US technology by itself had no measurable influence on the starch structure and properties. On the other hand, the ozone proved to be an effective technology to modify maize starch, alone or in combination with US. The ozonated samples (O₃, O₃-US and US-O₃) exhibited significant changes on the starch's molecular structure (carbonyl and carboxyl groups, pH, molecular size distribution and apparent amylose content) and properties (WAI, WSI, pasting properties, gel strength and paste clarity). On the other hand, the surface morphology, PSD and crystallinity of the maize starch samples were not affected by any treatment, alone or combined.

It is worth highlighting that the paste clarity was significant higher when a combination of the treatments was applied, especially when the US was used prior the O₃ treatment, illustrating the hypothesis that US changed the starch structure, improving the ozone action. However, further studies are needed to better understand the phenomena and optimize processing, comprising conditions of processing and starch sources.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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APPENDIX F: Irradiation of mung beans (*Vigna radiata*): a prospective study correlating the properties of starch and grains

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journal homepage: <http://www.elsevier.com/locate/ijbiomac>Irradiation of mung beans (*Vigna radiata*): A prospective study correlating the properties of starch and grains

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ABSTRACT

In this work, the effect of Gamma-irradiation was evaluated on the characteristics of mung bean (*Vigna radiata*) grains and starches, considering doses up to 5 kGy. For this purpose, the starch structure and properties were evaluated, as well as the grains' hydration, germination and cooking. The irradiation process was able to change the characteristics of both mung bean starches and grains. The starch structure was partially changed, presenting smaller molecules and small changes in the granule morphology. No alterations were observed in the starch X-ray diffraction pattern, while lower pH was achieved. Considering the starch properties, it was observed lower water retention ability at 75 °C, lower apparent viscosity, higher paste clarity and, in general, harder and less viscous gels. The ionizing radiation accelerated the hydration, reduced the germination capacity and improved cooking time of the mung bean grains. The results proved the efficacy of using ionizing radiation, at the doses applied in this work, to desirably modify the mung bean starch and grains.

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1. Introduction

Ionizing radiation is a high-energy technology, pointed as an effective and emerging technology with different applications in food processing. It is a physical treatment which involves a direct exposure of the product to ionizing electromagnetic rays, aiming to guarantee its safety and also improve its properties [1–3]. The principle of action of this method is based on the generation of free radicals, which are capable of causing the modification on the exposed materials [3,4]. In fact, after decades of study, the food irradiation was proved to be safe, being recognized as a food technology around the world [5].

However, although there is a vast literature regarding the use of irradiation for microbial inactivation, the effect of this technology on technological properties of foods still needs further studies. In special, Gamma-irradiation can be used to desirably modify food products and ingredients, although this approach needs further studies.

The hydration and germination of grains are two important steps of the grains industrialization. The ionizing radiation was reported to affect several of the seeds metabolics (growth and development), as well as its morphology (changing cells and tissues) [6]. The effect of the Gamma-irradiation on the hydration and germination of mung bean grains, however, is still unexplored. In fact, there is only one work on the literature evaluating the effect of irradiation on the hydration kinetics of grains (Ramaswamy et al. [7]). Even so, the work of Ramaswamy et al. [7] was carried out with fava beans, whose hydration

behaviour is different of other typical pulses [8]. Furthermore, the correlation between the observed behaviour of the grain and the properties of its starch was not carried out.

Further, the modification of starches is important to obtain ingredients with specific functional properties for their innumerable industrial applications. Most of the methods, however, are complex, time consuming and/or generates undesirable wastes [9]. Several works reported the use of Gamma-irradiation on the modification of different starch sources, as rice [10,11], chickpea [4], potato [12,13] and maize [14], among others. The results, however, do not always follow the same behavior, probably due to the different starch sources and/or irradiation dose. Furthermore, the irradiation of mung bean starch has not been evaluated yet.

In this work, the effect of Gamma-irradiation was evaluated on the characteristics of mung bean (*Vigna radiata*) grains and starches. For this purpose, the grains' hydration, germination and texture after cooking were evaluated, as well as the starch structure and properties. Furthermore, this work correlated the observed behaviour of the grain with the structure of properties of its starch. To the best of our knowledge, the effect of this technology on the mung bean grains and starches is firstly described here.

2. Material and methods

2.1. Material

Mung beans (*Vigna radiata*) were obtained at a local market of Campinas, SP, Brazil. This pulse presented $10.52 \pm 0.76\%$ d.b (g water/100 g

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of dry matter) of moisture content and average dimensions of 4.89 ± 0.42 mm length, 3.75 ± 0.34 mm width and 3.52 ± 0.11 mm thick.

For the starch extraction, the grains were hydrated at 5 ± 2 °C (to avoid germination) for 12 h and then milled with distilled water using a blender. The mixture was sieved using firstly a sieve of 60 mesh and then a sieve of 325 mesh, washing the supernatant with distilled water. The starch was separated from the water, protein and lipids by centrifugation (3200g for 5 min at 25 °C). Finally, the starch was dried at 35 °C until 10.3% of moisture content (~12 h) and then softly milled using a mortar and a pestle.

2.2. Irradiation process

The irradiation process was conducted at CENA/USP using a ^{60}Co radioisotope source. The samples were packed in 100 mL containers of polypropylene with a polyethylene screw cap and irradiated at ambient temperature (~25 °C). The grains (~60 g) and the starch (~40 g, 10.3% moisture content) samples were irradiated using different radiation dose up to 5 kGy. A non-irradiated sample (0 kGy) was used as control.

2.3. Starch evaluation

2.3.1. Granule morphology: scanning electron microscopy (SEM) and light microscopy

The surface and the morphology of the starch granules were evaluated using a scanning electron microscope (LEO 435 VP, Leo Electron Microscopy Ltd., Cambridge, England) with an acceleration voltage of 20 kV. The starch samples were sprinkled on double-sided adhesive tape placed over circular stubs and coated with a 30-nm gold layer.

The shape of the starch granules was evaluated using a light microscope (model L1000, Bioval, Curitiba, Brazil) with a 20-W halogen lamp. A 1% dispersion of starch in distilled water was mixed with a drop of Lugol solution (to better distinguish the granules). The dispersion was put onto a glass slide, which was covered by a glass cover slip, and observed in the microscope. The magnification used was of 400× and a portable camera of 1.3-megapixel was used to obtain the images.

2.3.2. Granule crystallinity: X-ray diffraction pattern and relative crystallinity

Firstly, to ensure a constant activity of water, the starch samples were maintained in a desiccator containing saturated BaCl_2 solution (25 °C, $a_w = 0.900$) for 10 days. After this period, the diffraction pattern of the samples was obtained using an X-ray diffractometer (Shimadzu XRD 7000, Tokyo, Japan) with copper radiation at an angle 2θ ranging from 3 to 40°. The working conditions were: scan rate of $2^\circ\cdot\text{min}^{-1}$, 40 kV and 30 mA. The curves obtained were smoothed using the Origin software, version 9.1 (Microcal Inc., Northampton, MA, USA), and the relative crystallinity (RC) of the starch granules was calculated as described by Nara e Komiya [15], using the same software, plotting the graphs between 2θ angles from 3 to 40°.

2.3.3. Molecular size distribution

The molecular size distribution profile of the starch molecules was determined by gel permeation chromatography (GPC). The methodology was based on the described by Song and Jane [16], with modifications. The samples were prepared as follow: 0.1 g of starch was mixed with 10 mL of Dimethylsulfoxide (DMSO; 90%, Labsynth, Brazil) and heated in a bath of boiling water for 1 h and then kept at 25 °C for 24 h under constant stirring. An aliquot of 3 mL of this solution was mixed with 10 mL of absolute ethanol and then centrifuged for 30 min at 3000g. The precipitated starch was mixed with 10 mL of boiling distilled water and heated until complete dissolution. 8 mL of this solution was then upwardly eluted in a glass chromatographic column (2.6 cm diameter and 70 cm high, packed with Sepharose CL-2B gel - Sigma, Sweden), with an eluent solution ($25 \text{ mmol}\cdot\text{L}^{-1}$ of NaCl and

$1 \text{ mmol}\cdot\text{L}^{-1}$ of NaOH), at a rate of $60 \text{ mL}\cdot\text{h}^{-1}$. A fraction collector (Gilson, model FC203B, Middleton, England) was used to separate the sample into 4-mL portions. Each portion was mixed with 0.1 mL of Lugol solution (blue value method, according to Juliano [17]), and evaluated using a spectrometer (Femto, Model 600S, São Paulo, Brazil) at 620 nm.

2.3.4. pH

The pH was determined using a calibrated potentiometer (Tecnal, TEC-5 mode, Piracicaba, Brazil), according to the specified by the Adolfo Lutz Institute [18]. A 10% starch slurry was maintained under constant stirring, using a magnetic stirrer, and then evaluated.

2.3.5. Pasting properties

The starch pasting properties were evaluated using a Rapid Visco Analyser equipment (RVA-4, Newport Scientific Pvt. Ltd., Australia, with the software TCW3). A suspension of 3 g (in relation to 14% moisture basis) of starch was homogenized in 25 g of distilled water for 10 s at 960 RPM and then heated and cooled under a constant shear (160 RPM). The suspension was initially held at 50 °C for 1 min, then heated to 95 °C at a rate of $6^\circ\text{C}\cdot\text{min}^{-1}$, then kept at 95 °C for 5 min, followed by cooling to 50 °C at a rate of $6^\circ\text{C}\cdot\text{min}^{-1}$, and finally holding it at 50 °C for 2 min. The apparent viscosity was recorded over the procedure.

2.3.6. Gel strength

The strength of the starch gel was determined by a uniaxial compression procedure, using a Texture Analyser (TA.XT Plus, Stable Micro Systems Ltd., Surrey, UK) with a load cell of 50 kgf (490.3 N). The gels obtained after the RVA assays were stored in plastic cups (40 mm diameter \times 20 mm height) for 24 h at 5 ± 2 °C inside a desiccator (to ensure uniform moisture of the samples and avoid drying). After the storage period, the gels were removed from the cups and compressed until the distance of 15 mm at $1 \text{ mm}\cdot\text{s}^{-1}$, using a cylindrical probe with 100 mm of diameter (SMS P/100). A drop of mineral oil was placed above and below the gel cylinder to ensure that only normal forces were measured by the probe.

2.3.7. Water Absorption Index (WAI) and Water Solubility Index (WSI)

The starch water absorption (WAI) and solubility (WSI) indexes were evaluated as described by Anderson et al. [19], with modifications. 0.5 g of starch (dry basis) were mixed with 6 mL of distilled water in pre-weighed centrifuge tubes. The tubes were then brought to water bath with stirring for 30 min at different temperatures (25, 50 and 75 °C). After heating, the tubes were centrifuged at 3000g for 10 min, and two phases were obtained: the supernatant and the precipitated.

The supernatant was dried at 105 °C in glass plates, and the soluble starch fraction (SS) of the supernatant was determined (Eq. (1)). Therefore, the WSI represents the soluble portion (%) of the starch at the given temperature. The precipitated (PT) sample retained in the tube was also weighed. The WAI is an indicator of the starch swelling power and represents the amount of water that 1 g of starch (disregarding the soluble portion) was able to absorb and retain (Eq. (2)). For both equations, MS is the mass of the starch sample, in dry basis.

$$\text{WSI} (\%) = \frac{\text{SS}}{\text{MS}} \cdot 100 \quad (1)$$

$$\text{WAI} \left(\frac{\text{g water}}{\text{g starch}} \right) = \frac{\text{PT} - (\text{MS} - \text{SS})}{(\text{MS} - \text{SS})} \quad (2)$$

2.3.8. Paste clarity

The starch paste clarity was evaluated as described by Craig et al. [20] and modified by Aplevicz and Demiate [21], by transmittance

(T%) measurement. 0.2 g of starch was mixed with 19.8 mL of distilled water in test tubes with screw caps. The tubes were then placed in a thermal bath with boiling water for 30 min and stirred individually every 5 min. The tubes were then cooled to room temperature and evaluated in a spectrometer at a 650 nm wavelength (Femto, Model 600S, São Paulo, Brazil).

2.4. Grain hydration process

The hydration process was conducted immersing approximately 10 g of grains in 250 mL of distilled water at 25 ± 1 °C. The temperature was controlled using a water bath (Dubnoff MA 095 MARCONI, Brazil). The hydration kinetics was obtained following the methodology described by Miano and Augusto [8], where the grain moisture content during soaking time is obtained by mass balance.

Since the hydration kinetics of mung bean presents sigmoidal behaviour, the data of moisture content (M_t) against soaking time (t) was fitted to the equation proposed by Kaptso et al. [22] (Eq. (3)).

$$M_t = \frac{M_\infty}{1 + \exp[-k \cdot (t - \tau)]} \quad (3)$$

where M_∞ is the equilibrium moisture content, k is the hydration rate and τ is the lag phase time of hydration (the required time to change the curve concavity).

2.5. Grain germination

The germination was studied by obtaining the germination courses (percentage of germination against time). For which, 50 seed were immersed in distilled water at 25 ± 1 °C using a Beaker. The temperature was controlled using a water bath (Dubnoff MA 095 MARCONI, Brazil). Every certain time, the germinated beans were counted. The germinated grain was considered when its radicle grows 3 mm outside the seed. The data of the percentage of germinated beans (G) against time (t) was tabulated and fitted to the sigmoidal function of Gompertz, modified by Zwietering et al. [23] (Eq. (4)).

$$G = G_{\max} \cdot \exp \left\{ - \exp \left[\frac{k_g \cdot e}{G_{\max}} (\lambda - t) + 1 \right] \right\} \quad (4)$$

where G_{\max} is the maximum germination percentage, k_g is the germination rate, λ is the lag phase time of germination (the required time to start the germination) and e is the Euler number.

2.6. Grain cooking kinetics

Native and irradiated beans were cooked in distilled boiling water (98.8 °C) for different periods (0, 5, 10, 15 and 20 min). In each of that periods, the texture of the grains were immediately evaluated through a uniaxial compression assay using a Texture Analyser (TA.XT Plus, Stable Micro Systems Ltd., Surrey, UK) with a load cell of 50 kgf (490.3 N). The grains were compressed until half of their width at a velocity of $1 \text{ mm} \cdot \text{s}^{-1}$ using a cylinder probe of 35 mm of diameter (P/35). The force measured by the equipment as a function of the compression was recorded, being the maximum peak considering for cooking description. It should be mentioned that the moisture content of the beans before being cooked was the same for all samples, to isolate the effect of irradiation from the effect of the initial moisture content. For that, according to their respectively hydration kinetics, the beans of each treatment were hydrated enough time to reach the same equilibrium moisture content of the control sample (25 °C).

The maximum force against the cooking time was plotted and fitted using Eq. (5) to estimate the maximum softening of the grains

[24]. Where F_0 is the force before cooking process, F_t is the force as function of cooking time, F_∞ is the minimum force that beans reached during cooking and k_f is the softening rate during cooking.

$$F_t = F_\infty + (F_0 - F_\infty) \cdot e^{-k_f \cdot t} \quad (5)$$

2.7. Experimental design, mathematical fitting and statistical evaluation

A completely randomized design was applied in three replicates. For the nonlinear regressions, each replication datum was fitted using a generalized reduced gradient algorithm, which is implemented in the 'Solver' tool in the Excel 2016 software (Microsoft, USA). Different initial guesses of the three parameters were assessed to detect possible local convergence. Furthermore, when relevant, a statistical analysis was performed on the treatments through analysis of variance (ANOVA) and Tukey's test, using the Statistica 12.0 software (StatSoft, USA). A significance level of 5% was considered.

3. Results and discussion

3.1. Starch structure evaluation

3.1.1. Granule morphology

The light microscopy and SEM images of the starch granules are shown in Fig. 1. Both native and irradiated starch presented oval-shaped granules with "cavities" in the central region. Few changes were observed in the surfaces of the irradiated samples, as slight imperfections (as pores). These imperfections could only be seen through the SEM images. In the 5 kGy sample, it was possible to find some broken granules, but in a very small quantity (as shown by the red arrow in the Fig. 1).

Gani et al. [25] studied the effect of the irradiation process up to 20 kGy in starches from 4 different kidney bean samples. They observed some granule disruption by the irradiation process, with increasing surface fracturing as the irradiation dose increased. The authors observed that the changes were apparently dose-dependent and related these results with the highly energetic and penetrating effect of the radiation. Other authors observed similar results for rice [9], beans and potato starches [26], as surface cracking, deformation of the granular structure and size decreasing.

On the other hand, Liu et al. [14] did not observe any visible changes in the shape, size or surface of irradiated maize starch granules, even after extremely high doses of gamma irradiation (500 kGy). The authors discussed that, in this case, the irradiation must have affected only the internal region of the granules, damaging the structure of the molecules.

Those results indicate that not only the irradiation dose, but also the starch intrinsic characteristics, interfere in the irradiation process. Considering the mung bean starch samples, where the external structure of the granules was affected, it is possible to state that the irradiation affected both internal and external structures.

3.1.2. Molecular structure

Fig. 2 represents three different aspects of the starch internal structure: the size distribution of starch molecules (Fig. 2A), the pH of the starch suspensions (Fig. 2B) and the granule X-ray diffraction patterns and relative crystallinity (Fig. 2C and D). These properties would indicate, respectively, if there was hydrolysis of the glycosidic bonds of the molecules (leading to a formation of small-sized chains), if there was oxidation with possible formation of acid groups (which decreases the pH of the samples) and if there was any change in the crystalline structure of the starch samples.

Fig. 2A shows the starch molecular size distribution. In the gel permeation chromatography analysis, a gel with known porosity is used,

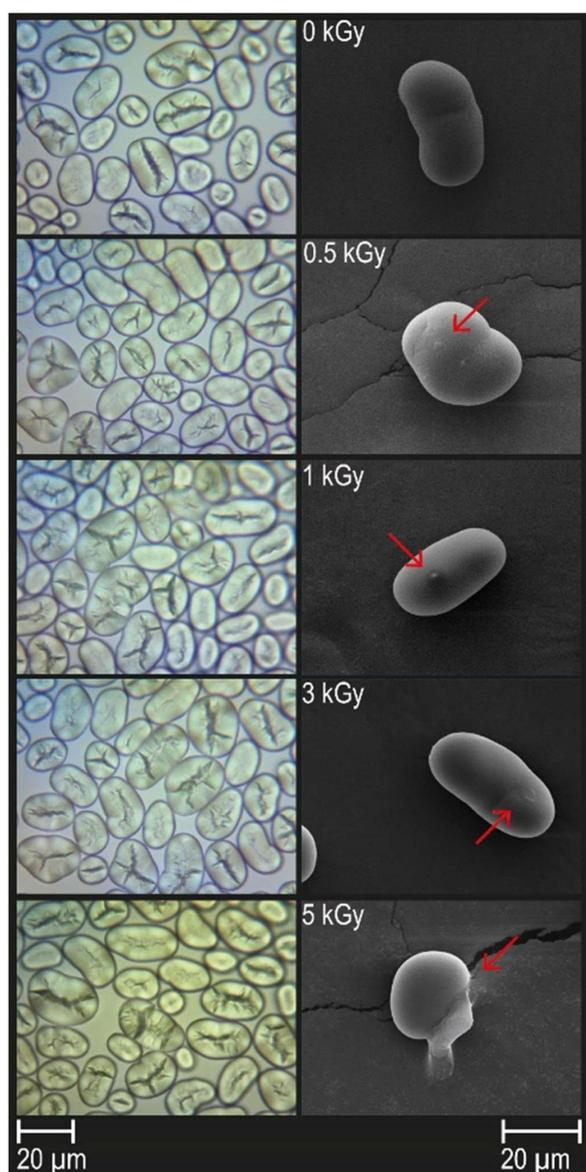


Fig. 1. Light microscopy (left column) and scanning electron microscopy (right column) of the native (0 kGy) and irradiated (0.5, 1, 3 and 5 kGy) mung bean starch. The white lines measure 20 μm . The red arrows indicate imperfections in the granules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the smaller molecules can penetrate the pores and be retained longer in the chromatographic column. In this way, the larger and more branched molecules (as is the amylopectin) usually elute first, composing the first peak of the chromatogram, being followed by the small-sized fractions [27]. With increasing irradiation dose, both first and second peaks apparently “increased” in all samples (except the first peak of the 5 kGy sample). Also, it could be observed an “intermediate” peak in the 0.5 and 1 kGy samples (as a “shoulder” of the second peak). These results can indicate two main factors: the gradual size-decreasing of the starch molecules (eluting later in the chromatographic column)

and the possible formation of molecular fractions with higher iodine affinity (i.e. less branched).

It is possible to say that the low-dose irradiated samples (0.5 and 1 kGy) suffered a light hydrolysis, which was able to generate molecules with smaller chain-length and higher iodine affinity. However, after a more intense irradiation (3 and 5 kGy), the molecules were eluting after the second peak or were not able to complex the iodine anymore due to their small chain length. It was illustrated by the displacement of the first and second peaks as the irradiation dose increases, with the rising and posterior “disappearance” of an intermediate “fraction”.

Another important aspect is that the first peak, which is known as the “amylopectin peak” (as previously explained), was not completely degraded. This indicates that the irradiation process do not completely destroy the amylopectin chains at this conditions (up to 5 kGy), differently as occurs after severe oxidative process, for example [28].

In the literature, several authors [4,13,26,29] reported the hydrolysis of the glycosidic bonds as one of the consequences of the irradiation process of starches, leading to a decrease in their chain length. These results can be related to the formation of free radicals on the starch molecules, as discussed by Sokhey and Hanna [29]. Summarizing, the chromatographic results can prove the molecular depolymerization of the mung bean starch samples after the irradiation process, being in accordance with the reported in the literature. It can affect both starch and grain properties.

Fig. 2B indicates that the starch suspension pH value decreased with increasing irradiation process. The pH was used as an indirect measurement of acid groups' formation [12]. Similar trend was reported by other authors [12,25,30,31], which associated the pH decreasing with the formation of carbonyl and carboxyl groups in the irradiated starches, as well as the formation of different acids (acetic, formic, pyruvic and glucuronic). The results indicate, therefore, that the irradiation process contributed to the oxidation of the hydroxyl groups of the starch samples, especially into carbonyl and carboxyl groups. Besides, as the pH decreased, we can suppose that the presence of the acid groups was higher in the most irradiated dose.

The X-ray diffraction pattern of mung bean starch can be observed in Fig. 2C, and their respective Relative Crystallinity (RC) in Fig. 2D. The X-ray diffraction pattern indicate that the mung bean starch can be designated as C-type pattern, according to the classical literature [32,33].

However, a current approach is being used to evaluate the starch diffraction patterns, and it is based on the comparison of the X-ray results with a database of Powder Diffraction Files (PDF-4) [34,35]. This database is still under construction, and thus present only the amylose crystalline structure catalogued. According to this approach, we can observe that the peaks of the mung bean starch samples were located at $\sim 15^\circ$, 17° , 18° and 23° in both native and irradiated samples. This indicates that the irradiation did not changed the crystalline pattern of the amylose and the amylopectin molecules.

The RC was not significant affected ($p < 0.05$) by the irradiation process (Fig. 2D), indicating that, despite the higher proportion of small molecules observed in the chromatography, the crystallinity of the samples was not affected by the irradiation (as the intensity of their X-ray diffraction pattern did not change). Besides, the amylose molecules seem to be as affected as the amylopectin molecules by the process, since the displacement of both peaks were similar in the chromatography. This can indicate that the irradiation process uniformly affects the molecules within the starch granules.

These results are in accordance with the literature, where the irradiation process is described to degrade the starch molecules through the generation and transformation of free radicals [9], that are capable of changing both internally and externally the molecules. This is also supported by the fact that the surface of the starch granules presented little or no changes (Fig. 1).

To sum up, the starch structural changes can be summarized as: smaller molecules (indicating the hydrolysis of the glycosidic bonds), lower pH (indicating acid groups formation), little changes in the granule morphology and no alterations in the diffraction pattern of the starch samples.

In the next section it will be discussed how the structural changes affected the starch technological properties.

3.2. Starch properties evaluation

Fig. 3 presents the starch Water Absorption Index (WAI, Fig. 3A), Water Solubility Index (WSI, Fig. 3B), pasting properties (RVA, Fig. 3C) and paste clarity (Fig. 3D) as a function of irradiation dose.

Regarding the WAI results it is possible to observe that, at 25 and 50 °C, no significant difference was observed among the samples. This result is of great importance to understand the hydration process behaviour (discussed later in this work), since the hydration behaviour of the grains at 25 °C was affected by the irradiation, and some authors hypothesized that this behaviour was related to the increase of the starch water absorption (which was not). On the other hand, the WAI of the irradiated starch were lower at 75 °C than the native one ($p < 0.05$).

These results indicate that the WAI of the starch granules below the gelatinization temperature was not affected by the irradiation – in fact because that values are very small, once the water absorption by the granule at that conditions is negligible. On the other hand, at 75 °C the structural changes undergone by the most irradiated samples (3 and 5 kGy) were sufficient to affect their water absorption and retention capacity. This can be explained by the lower molecular size of the irradiated starches and, especially, by the replacement of the hydroxyl groups by other less hydrophilic groups.

The WSI (Fig. 3B) did not differ significantly from the native starch sample, in any observed temperature. In this case, the structural changes undergone by the starch molecules after the irradiation was

not sufficient to change their solubility, in conditions applied in this work.

Regarding the pasting properties (Fig. 3C), it is possible to observe that irradiation reduced the paste apparent viscosity, if compared to the native starch. The same behaviour was reported by Chung & Liu [26] while studying potato and beans starches irradiated up to 50 kGy, and by Gul et al. [11] while studying irradiated rice starch up to 10 kGy. The decrease in the apparent viscosity indicates that, under the RVA analysis conditions (i.e. stirring and heating), the modified starch presented a reduced capacity to maintain their integrity, if compared to the native sample. The granule is broken in a lower temperature. This result can be explained by the cleavage of the glycosidic bonds of the starch molecules, which decreases the size of their chains, resulting in “weaker” granules.

The paste clarity (Fig. 3D) increased with increasing the irradiation dose. The 5 kGy sample reached almost the double of the transmittance observed for the native sample. These results are in accordance with the structural changes undergone by the irradiated samples. The carboxylic groups are electronegative and cause an electrostatic repulsion between the molecules, impairing their association [36]. The less intra and intermolecular associations, the higher will be the paste clarity [20]. It is worth mention that this is a very positive change, considering industrial application.

Fig. 4 illustrates the mechanical properties of the mung bean starch at different irradiation dose. The starch gels were evaluated through a compression test, until their complete disruption. The obtained results can be discussed using the relative strain that the samples reached before their disruption (Fig. 4B), as well as by comparing the peak force that the samples were able to reach before their disruption (Fig. 4C). It is possible to observe that the native sample was able to deform almost ~65% of its height before its disruption, a value much higher than the 5 kGy irradiated sample (which reached ~50% of its height before disrupting). The 0.5, 1 and 3 kGy samples were statistically ($p > 0.05$) equals to the native sample, besides presenting lower values of relative

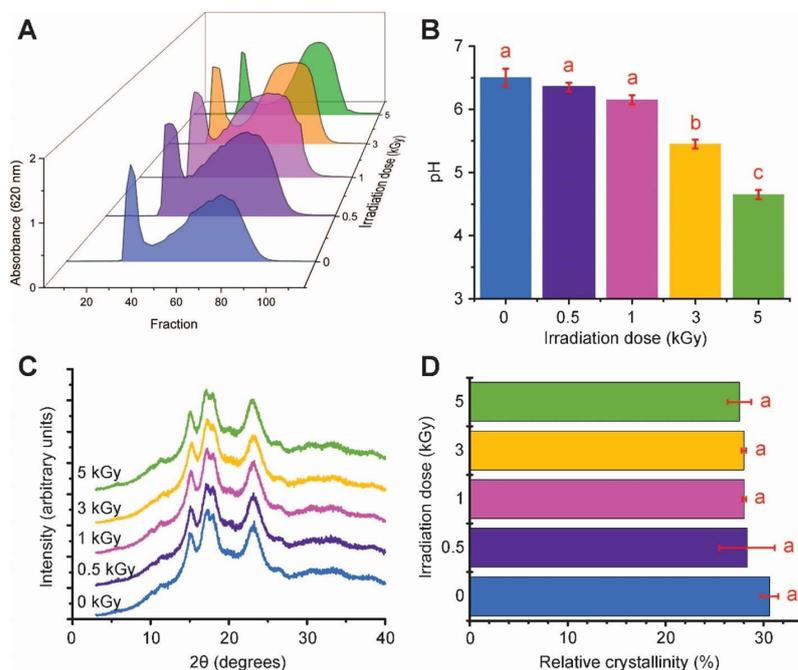


Fig. 2. (A) Molecular size distribution profile (blue value method), (B) pH values, (C) X-ray diffraction patterns and (D) relative crystallinity of the native (0 kGy) and irradiated (0.5, 1, 3 and 5 kGy) mung bean starch. Red bars indicate the standard deviations. Variations followed by the same small letters do not differ significantly ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

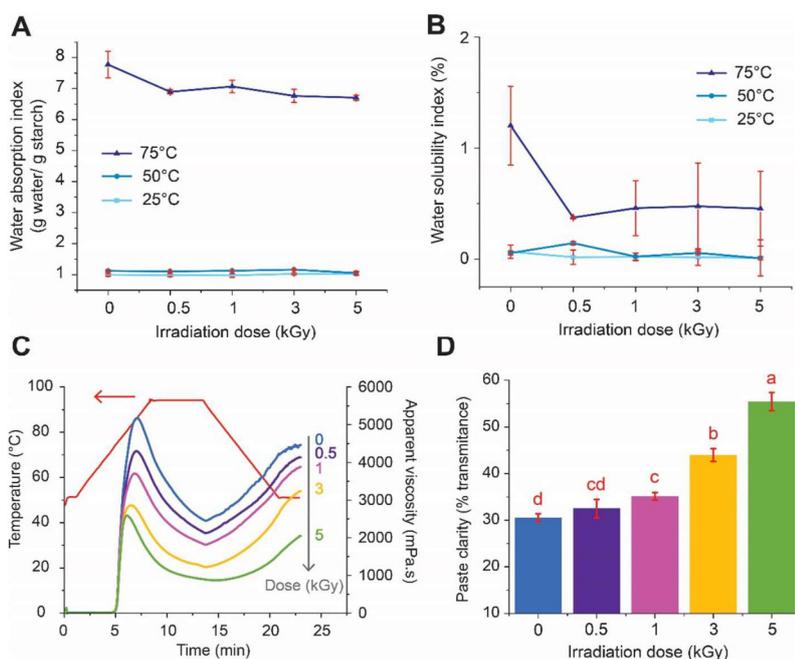


Fig. 3. (A) Water absorption index, (B) water solubility index, (C) pasting properties and (D) paste clarity of the native (0 kGy) and irradiated (0.5, 1, 3 and 5 kGy) mung bean starch. Red bars indicate the standard deviations. Variations in the properties due to irradiation dose, when followed by the same small letters, do not differ significantly ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strain. By comparing the peak force, the results show that the 1 and 3 kGy samples presented a stronger gel if compared to the native and to the 0.5 and 5 kGy samples. It is worth mention that a stronger gel, considering the same starch concentration, is a characteristic of high industrial relevance.

These results can be explained by the cleavage of the glycosidic bonds of the starch molecules. The lower chain-length molecules can present a size that is more suitable for re-association after cooling, thus constituting harder gels (that need more energy to disintegrate). However, these same molecules lead to gels that are not resistant to compression forces, being disrupted in a lower temperature if compared to the native starch gels.

Summarizing, the irradiation process led to starches with lower water retention ability, lower apparent viscosity, higher paste clarity and, in general, harder and less elastic gels. These characteristics can be very promising from an industrial point of view, especially industries like food, paper and textile, where a high paste clarity and a lower apparent viscosity are desirable.

3.3. Hydration process

The hydration kinetics of mung beans showed a sigmoidal behaviour (Fig. 5), as expected [37]. This behaviour is usually found on grains from *Phabaceae* family, which it is characterized by a lag phase caused by the seed coat impermeability to water when at small activity of water values [8]. In fact, the hydration kinetics of seeds during all germination process produces a triphasic curve [38]: the first phase consists of the actual hydration process; in the second phase the moisture content of the seed keeps constant and the nutrients catabolism takes place; in the third phase the radicle grows to form the seedling and the moisture content is increased due to the cell reproduction (Fig. 5).

The model proposed by Kaptso et al. [22] was used to describe the hydration kinetics. As mung beans have a very rapid germination, the second phase of the curve is very short, and the phase 1 and phase 3 seems to be joined (Fig. 5, Miano et al. [37]). Consequently, it is difficult to determine the equilibrium moisture content during the hydration process of mung beans. Therefore, the equilibrium moisture content

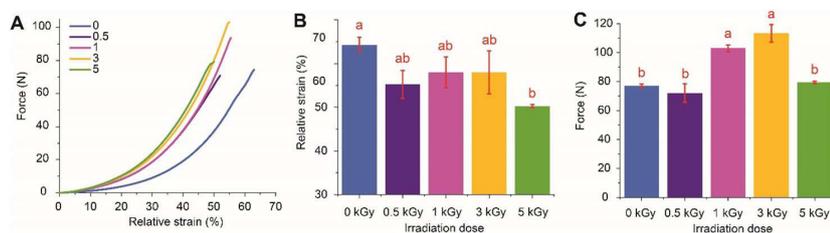


Fig. 4. (A) Gel compression force vs. relative strain curves until the peak force; (B) Relative strain of the peak force and (C) peak force of the native (0 kGy) and irradiated (0.5, 1, 3 and 5 kGy) mung bean starch. Red bars indicate the standard deviations. Variations followed by the same small letters do not differ significantly ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

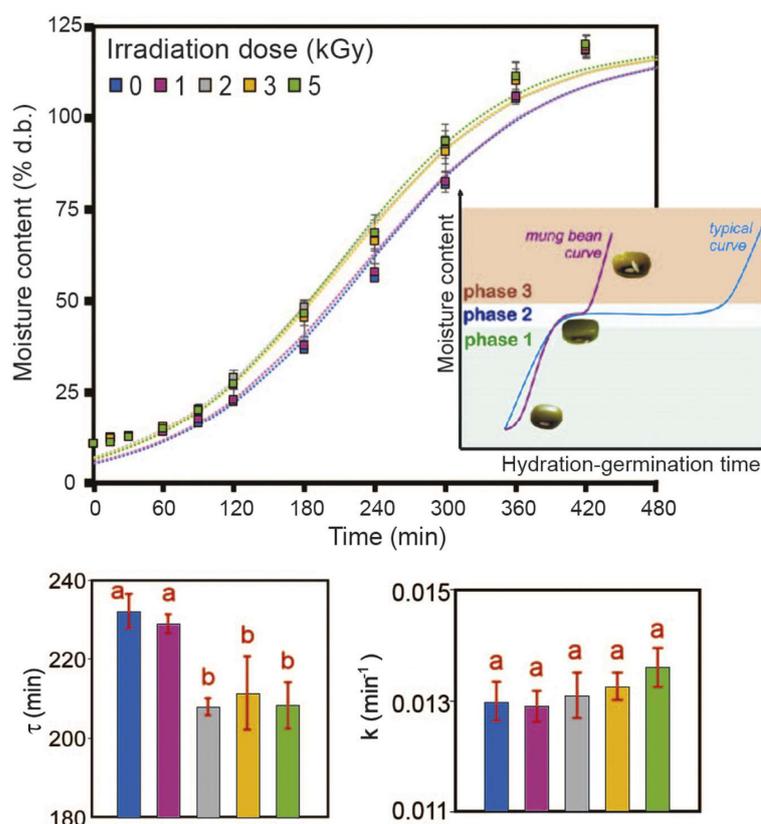


Fig. 5. Up: Hydration kinetics and the relation between moisture content and hydration-germination process of a typical seed and of mung bean seeds. Note the short phase 2 of mung bean due to its fast germination. Down: Effect of gamma radiation on the hydration kinetics of mung bean: Effect on the lag phase time (τ) and hydration rate (k). The dots represent the mean of the experimental data, the lines represent the model (Kaptso et al., 2008) and the vertical bars represent the standard deviation. Lowercase letters represent Tukey mean comparison test (95% of confidence).

(M_∞), was considered the same for all the treatment, as the average value of all treatments, as $119 \pm 1\%$ d.b. The hydration rate (k) and the phase lag time (τ) were determined individually for each treatment. As shown in Fig. 5, this approach successfully fit the data for all the used irradiation doses ($R^2 > 0.99$).

The ionizing radiation affected significantly ($p < 0.05$) the hydration kinetics of mung beans. According to Fig. 5, when 2, 3 and 5 kGy were applied, the hydration process was faster. In fact, the acceleration was due to the reduction of the lag phase (represented by τ), since the hydration rate (k) was not significantly affected (Fig. 5).

The parameter τ is related to the necessary time to increase the seed coat water activity in order to change its permeability to water [39]. At low moisture contents, the seed coat of many pulses is impermeable to water. Therefore, water enters the grain by the hilum and/or micropyle, hydrating the seed coat from inside until increasing its permeability to water [8].

In fact, the most probably cause of the lag phase reduction would be changes in the seed coat components. The seed coat is composed by carbohydrates and some components such as phytic acid, tannins and phenolics compounds [40]. These components could be affected by ionizing radiation, facilitating the seed coat hydration and changing its permeability faster. For instance, some works reported reduction of tannins and phytic acid on common beans using irradiation doses up to 10 kGy [41] and on fava beans using 1 kGy [42]. Therefore, the modification of these components may affect the permeability of the seed coat.

On the other hand, the hydration rate (k) was not affected by irradiation. This parameter is related to the velocity of hydration of the

cotyledon and germ and represent a global water transfer constant, considering both diffusion and capillarity mechanisms [8]. Furthermore, the curve shape keeps very similar, without big differences. This probably means that irradiation did not significantly affect the cotyledon composition, and that its main effects take place on the seed coat.

In fact, despite the ionizing irradiation improved the hydration process, this acceleration was short. Therefore, this technology would not be used for this purpose, since there are other more efficient technologies which accelerate hydration process, such as using high temperatures [43,44], high pressures [45] or ultrasound technology [37,46].

On the other hand, this prove that ionizing irradiation can be used for disinfestation and/or disinfection purposes on this grain without affecting negatively the hydration process. This is interesting and important considering different applications, such as for cooking and starch extraction.

Ramaswamy et al. [7] evaluated the effect of irradiation on the hydration kinetics of fava beans, whose hydration kinetics showed downward concave shape behaviour. For the best of our knowledge, this is the only study regarding irradiation and the hydration kinetics of grains. They found that the hydration rate was increased by using ionizing irradiation at doses of 2 and 5 kGy. The authors hypothesized that this behaviour was related to the increase of the starch water absorption. However, contrary to the present work, this property was not evaluated there. In the present work, we demonstrated that the water absorption of starch at 25 °C was not affected by irradiation (please verify the next sections), being important only at 75 °C.

Therefore, the acceleration of fava beans hydration [7] should be caused by other reason.

Further than the isolated components, it is important to evaluate the grain as complex material, with tissues and cells. Therefore, another probable explanation for the effect of irradiation can be the grain metabolism.

The ionizing radiation can change the cells metabolism, from membrane damage to the cell dead. Therefore, the irradiated cells could lose their biological control of water transfer, letting a faster water entrance. This hypothesis can explain the hydration enhancement due to the irradiation process. In fact, the changes on the grain metabolism can be demonstrated through its germination.

3.4. Germination process

Fig. 6A shows the effect of irradiation on the germination courses of mung beans. Ionizing irradiation affected significantly ($p < 0.05$) the germination. In fact, this pulse manages to germinate even at doses up to 5 kGy, demonstrating to be a resistant organism. Even so, it was perceived that the seedling has difficulties to develop when they were let to grow (results did not register). In general, the higher the irradiation dose was, the lower the germination capacity was. This was because ionizing irradiation causes mutation on mung bean cells [47]. Therefore, this probable avoids some enzyme formation for reserve catabolism and seedling development.

As the germination kinetics presents a sigmoidal behaviour, the model of Gompertz was used to fit the data. Among different parametrizations, that one modified by Zwietering et al. [23] was adopted (Eq. (4)). This equation provided three parameters that helped to study the effect of irradiation on the germination process: the

maximum germination (G_{max}), the germination rate (k_g), and the lag phase time (λ).

Fig. 6B shows that irradiation affected significantly the maximum grain germination (G_{max}). In fact, the maximum germination percentage significantly decreased ($p < 0.05$) only when 5 kGy were applied, from ~95% to ~72%. Despite this result, mung bean has shown a high resistance to ionizing irradiation comparing to other seeds. Many other seeds have demonstrated a great reduction on their maximum germination count when ionizing irradiation was applied. For instance, the germination capacity of wheat seed was null when 0.6 kGy was applied [48]; the germination capacity of *Lathyrus chrysanthus* was reduced to the half when 0.25 kGy were applied [6]; and the germination capacity of chick peas was reduced 40% when 1 kGy was applied [49].

The germination rate (k_g) was also significantly affected by ionizing radiation (Fig. 6C). In this case, the germination rate was reduced approximately 40% when 1, 2 or 3 kGy were applied, and approximately 75% when 5 kGy were applied. Therefore, despite having a high maximum germination, the velocity of germination was sharply reduced. This was probably caused by the cell mutation and/or enzyme inactivation that causes the reduction of metabolism and reserve catabolism for the radicle to grow. On the other hand, the lag phase time (λ) was not significantly affected by irradiation, despite there is a slightly increment when applying 5 kGy (Fig. 6D). It should be mentioned that the value of the lag phase of germination (λ) is very similar to value of the lag phase of hydration (τ). In fact, the lag phase of hydration is related to the time when the seed coat components suffer glassy state transition (pass from glassy to rubbery state) [39,50]. Therefore, there is an increase of water mobility, which would help enzymes to be activated promoting the radicle growth. Consequently, both parameters are related.

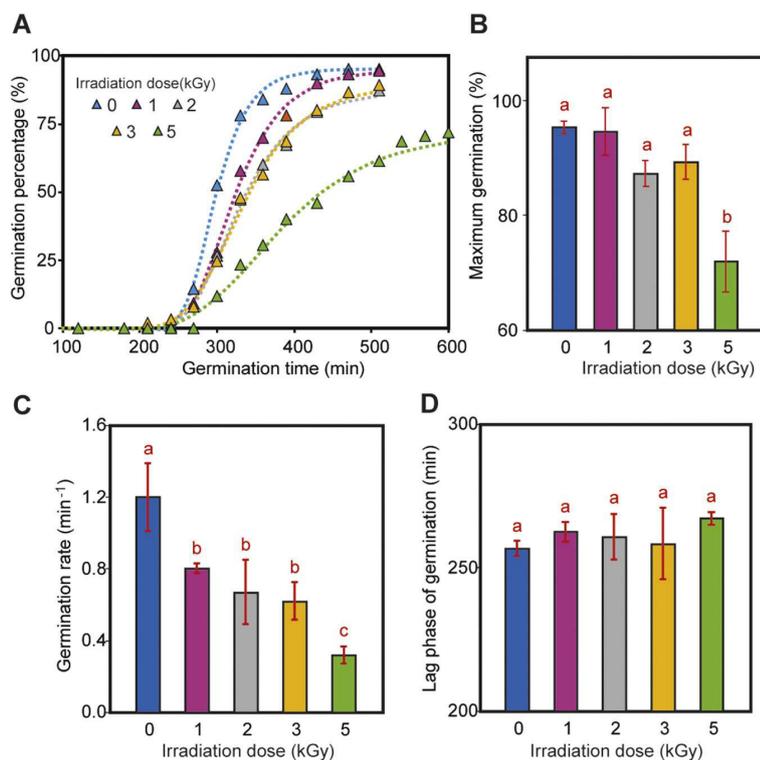


Fig. 6. Effect of gamma radiation on: (A) germination course of mung bean, (B) maximum germination percentage (G_{max}), (C) germination rate (k_g) and (D) lag phase of germination (λ). The triangles represent the mean of the experimental data, the lines represent the model of Gompertz (Eq. (4)) and the vertical bars represent the standard deviation. Lowercase letters represent Tukey mean comparison test (95% of confidence).

Summarizing, if the purpose of mung bean is sprouting, the irradiation would not be useful. However, if the purpose is to avoid the germination, besides de disinfestation and disinfection, for cooking and starch extraction, irradiation would be an excellent approach.

3.5. Grain cooking kinetics

The cooking kinetics of mung bean was evaluated through compression assays during cooking. The results are shown in Fig. 7, including the parameters of the kinetics model. The initial and equilibrium forces (Fig. 7B) of the mung bean samples did not present a statistical difference ($p < 0.05$). Similarly, the cooking kinetics parameter did not present a statistical difference ($p < 0.05$, Fig. 7C), although a clear trend can be observed (Fig. 7A and C).

After 5 min of cooking time (Fig. 7A), it is possible to see a difference among the values of force presented by the irradiated ($2.3 \text{ kGy} = 8.2 \pm 0.3 \text{ N}$; $3.8 \text{ kGy} = 7.6 \pm 0.8 \text{ N}$) and the control ($0 \text{ kGy} = 11.1 \pm 0.4 \text{ N}$) samples. In fact, the irradiated samples reached values of force close to the equilibrium force ($\sim 3.5 \text{ N}$) 5 min before the control sample, that reached the value of $5.5 \pm 1.6 \text{ N}$ only after 15 min of cooking.

Considering the results, it is possible to say that the irradiation led to a decrease in the time needed to cook the grains (i.e., the time needed until they reach the equilibrium force on Fig. 7A). It is worth to mention that this is a highly desirable result from an economic point of view, once reducing cooking time is one of the main objectives on bean development. Similar results (reduction of cooking time) were reported by Iyer et al. [51] using 5 kGy to irradiate Great Northern kidney and pinto beans.

Since the irradiation process is highly penetrating [52], the results can be explained by the partial degradation of both internal and external major components of the grains, as the starches and proteins, or even parts of the grain tissues and cells. In fact, the starch of the mung bean was highly affected by the irradiation process, as previously discussed in the present work. It is important to point out, however, that the observed changes in the cooking kinetics of the grains are also related to the higher temperatures applied for their cooking, since at lower temperatures (as demonstrated for water absorption capacity at 25°C , Fig. 3B) the possible changes caused by the irradiation process were not measurable.

Considering the effect of the irradiation on other components of the grains, two main reactions can be associated with the observed changes: the partial denaturation of the proteins, especially the ones presented in the grains' coats [53], and the changes in the phytate molecules (reduction of phytic acid by irradiation was reported by several authors, as Brigide & Canniatti-Brazaca [41], Osman et al. [42] and Villavicencio et al. [54]).

Both changes can be associated with a structure disruption and/or with the phytate bonds with other components, causing a further reduced cooking time of the mung bean grains.

The decrease in the cooking time achieved after the irradiation process can be useful in cases when the cooking of the grains is the limiting of a process.

4. Conclusions

The irradiation process, at the doses applied in this work, was able to change the characteristics of both mung bean grains and starches.

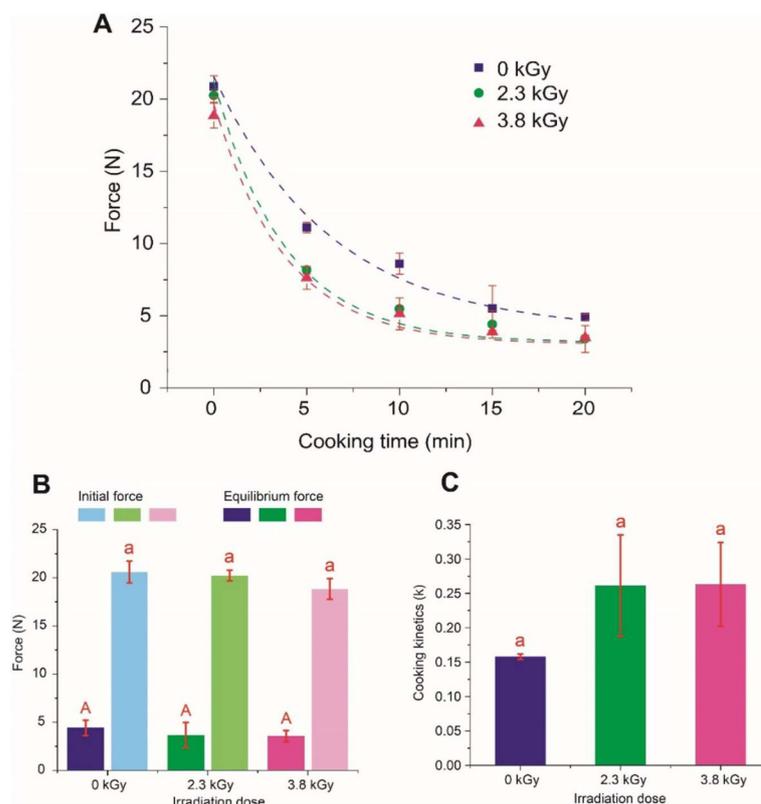


Fig. 7. Effect of gamma radiation on beans (A) cooking kinetics curves, (B) initial and equilibrium force of compression and (C) cooking kinetics (k). The symbols represent the mean of the experimental data, the dot lines represent the model of Eq. (5), and the vertical red bars represent the standard deviations. Same letters represent Tukey mean comparison test (95% of confidence). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Considering the starch samples' characteristics, their structure was partially changed, presenting smaller molecules, lower pH and little changes in the granule morphology. However, no alterations were observed in the X-ray diffraction pattern of the starch samples. Considering the starch properties, almost all of them underwent significant changes, except for the WAI at 25 and 50 °C and the WSI. It was observed lower water retention ability at 75 °C, lower apparent viscosity, higher paste clarity and, in general, harder and less elastic gels. The results proved the efficacy of using ionizing radiation on the modification of the mung bean starch, with possible industrial applications.

Considering the grains characteristics, ionizing radiation slightly accelerated the hydration of mung beans, reducing the lag phase time using doses of up to 5 kGy. These changes could not be attributed to the starch, being thus related with changes on the grain structure and/or metabolism. On the other hand, germination capacity was reduced up to 72% of germinated seeds using 5 kGy, proving a great resistance of mung bean to irradiation. The cooking time of the irradiated grains was also improved. Therefore, irradiation could be used for insects and microorganisms' control without affecting negatively the hydration process. Nevertheless, if sprouting is the main purpose, irradiation would not be useful.

Conflict of interest

The authors have declared no conflict of interest.

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APPENDIX G: Resumo simples/ Simple abstract/ Resumen sencillo

Resumo simples

Os amidos são substâncias encontradas abundantemente na natureza, e são muito utilizados para diversas aplicações industriais. Muitas vezes, entretanto, esses amidos são modificados para melhorar seu desempenho, o que pode envolver o uso de produtos químicos e conseqüentemente acarretar geração de resíduos que podem ficar no produto e/ou no meio ambiente. De forma a garantir um bom desempenho industrial para os amidos, porém utilizando métodos mais eficientes, seguros e/ou ambientalmente amigáveis, esta Tese explorou diferentes abordagens, utilizando tanto amidos em sua forma nativa (não modificada) quanto amidos modificados. Considerando os amidos nativos, uma fonte de amido pouco explorada (raiz de arracacha) foi caracterizada. Além disso, amidos comumente utilizados na indústria foram melhor avaliados utilizando metodologias de análise não convencionais. Considerando os amidos modificados, foram utilizadas tecnologias emergentes e com menor geração de resíduos: ozônio (um gás altamente oxidante), ultrassom (tecnologia que envolve uso de ondas acústicas de alta potência) e irradiação (uma energia ionizante). Cada uma das tecnologias apresentou vantagens e desvantagens. Porém, de modo geral, elas apresentaram grande potencial para serem utilizadas para modificar amidos industrialmente.

Simple abstract

Starches are abundantly found in nature, and are widely used for various industrial applications. However, starches are often modified to improve their performance, which may involve the use of chemicals and consequently generate wastes that may remain in the product and/or in the environment. In order to ensure good industrial performance for starches, but using more efficient, safer and/or environmentally friendly methods, this Thesis explored different approaches, using both native (unmodified) and modified starches. Considering native starches, an unexplored starch source (arracacha root) was characterized. In addition, starch sources that are commonly used in industry were better evaluated using unconventional analyses' methodologies. Considering the modified starches, emerging technologies with less waste generation were used: ozone (a highly oxidizing gas), ultrasound (a technology that involves the use of high power acoustic waves) and irradiation (an ionizing energy). Each of the technologies had advantages and disadvantages. However, in general, they presented a great potential to be used to industrially modify starches.

Resumen sencillo

El almidón es una sustancia que se encuentra abundantemente en la naturaleza y se usa ampliamente para diversas aplicaciones industriales. Sin embargo, a menudo, el almidón se modifica para mejorar su funcionalidad, lo que puede implicar el uso de productos químicos y, en consecuencia, generar residuos que pueden permanecer en el producto y/o el medio ambiente. Con el fin de garantizar la funcionalidad deseada en aplicaciones industriales para el almidón, pero utilizando métodos más eficientes, seguros y/o amigables con el medio ambiente, esta Tesis exploró diferentes enfoques, utilizando almidón nativo (no modificado) y modificado. Como almidón nativo, se caracterizó una fuente de almidón poco explorada (raíz de arracacha). Además, el almidón comúnmente utilizado en la industria se evaluó detalladamente utilizando metodologías de análisis no convencionales. Para producir almidón modificado, se utilizaron tecnologías emergentes: ozono (un gas altamente oxidante), ultrasonido (tecnología que implica el uso de ondas acústicas de alta potencia) e irradiación (una energía ionizante). Cada una de las tecnologías mencionadas presenta ventajas y desventajas. Sin embargo, en general, tienen un gran potencial para ser utilizadas para modificar almidón a nivel industrial.