University of São Paulo "Luiz de Queiroz" College of Agriculture

Starch modification using green technologies

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

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Starch modification using green technologies

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"Come to me, all you who are weary and burdened, and I will give you rest. Take my yoke upon you and learn from me, for I am gentle and humble in heart, and you will find rest for your souls. For my yoke is easy and my burden is light" (Matt. 11:28-30).

"But he said to me, 'My grace is sufficient for you, for my power is made perfect in weakness'. Therefore I will boast all the more gladly about my weaknesses, so that Christ's power may rest on me. That is why, for Christ's sake, I delight in weaknesses, in insults, in hardships, in persecutions, in difficulties. For when I am weak, then I am strong" (2 Cor. 12:9,10).

"But those who hope in the LORD will renew their strength. They will soar on wings like eagles; they will run and not grow weary, they will walk and not be faint" (Isa. 40:31).

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RESUMO Modificação de amidos através de tecnologias verdes

Amidos são ingredientes naturais amplamente utilizado em diferentes indústrias e tipos de produtos devido às suas diversas propriedades. No entanto, amidos nativos podem não apresentar propriedades específicas que são necessárias para uso industrial. Afim de superar limitações, a modificação de amidos é uma alternativa para melhoria de performance dos mesmos. Entretanto, a maioria dos métodos de modificação utilizados gera resíduos que devem ser adequadamente tratados para não comprometer o ambiente e os alimentos. Portanto, é essencial encontrar alternativas para suprir as necessidades da indústria sem gerar resíduos tóxicos. Nesse contexto, destaca-se a importância de estudar tecnologias emergentes com apelo ambiental, também chamadas de "tecnologias verdes", como o ozônio (O₃) e o aquecimento à seco (dry heating, DHT), sendo o objetivo deste trabalho. Esta Tese de Doutorado divide-se em cinco capítulos: O primeiro capítulo aborda o contexto deste trabalho, compreendendo as seções Introdução e Organização da Tese, Objetivos e Revisão da Literatura. Em seguida, no segundo e terceiro capítulos, avaliou-se o processo de ozonização em duas fontes de amido: mandioca (Capítulo 2) e arracacha (Capítulo 3). Nestes capítulos, foi demonstrado que o tratamento com ozônio foi capaz de formar grupos carbonila e carboxila, além de causar alterações na distribuição do tamanho molecular (despolimerização), alterando as propriedades dos amidos. Destacam-se algumas alterações com potencial aplicação na indústria, tais como redução da viscosidade aparente da pasta, redução da retrogradação do amido, géis mais fortes (inclusive em condições ácidas) e maior claridade de pasta. Em seguida, no Capítulo 4, o amido de mandioca foi modificado utilizando a combinação dos tratamentos com O₃ e DHT. Neste capítulo, percebeu-se que a combinação de tratamentos promoveu diferentes modificações em relação aos tratamentos individuais. Além disso, a sequência desses tratamentos influenciou muitas propriedades do amido modificado obtido. Por exemplo, as amostras que foram modificadas com a sequência de combinação de DHT com O₃ (DHT+O₃) apresentaram o maior grau de oxidação e um gel mais resistente à guebra do gue os demais géis. Por outro lado, o amido modificado com a sequência oposta de combinação (O3 + DHT) exibiu um grau de oxidação semelhante ao amido tratado com O₃, uma distribuição de tamanho molecular com moléculas de tamanho muito pequeno e capacidade de formar géis mais fracos do que o nativo. Por fim, a última parte da Tese (Capítulo 5) compreende as seções Conclusões Gerais e Sugestões para Trabalhos Futuros. Portanto, a relevância da presente Tese baseia-se no estudo aprofundado do processo de ozonização, bem como na proposta inovadora de combinar os processos de ozonização e DHT, com foco na discussão de alternativas "verdes" para a modificação do amido.

Palavras-chave: amido, modificação, ozônio (O₃), aquecimento a seco (*dry heating* - DHT).

ABSTRACT Starch modification using green technologies

Starches are widely used in different industries and products, due to their diverse properties. However native starches may not present some particular properties required for industrial use. In order to overcome limitations, the modification of starch is an alternative to improve their performance. However, most of starch modification methods used by the industry generate wastes that must be adequately treated to do not compromise the environment nor food. Therefore, alternatives to meet the industries' necessities without generating toxic residues are needed. In this context, it is important to study emerging "environmentally friendly" technologies, also called "green technologies", such as ozone (O_3) and dry heating treatments (DHT). This PhD Thesis is divided in five chapters: The first chapter contextualize this work, comprising Introduction and Thesis Organization, Objectives and Literature Review sections. Then, the ozonation process was evaluated in two starch sources: cassava (Chapter 2) and arracacha (Chapter 3). These chapters demonstrated the ozone treatment was able to form carbonyl and carboxyl groups, as well as to cause changes in the molecular size distribution (depolymerization), which affected and changed both cassava and arracacha starches properties. Some highlighted results with potential application are: reduction of the paste apparent viscosity, reduction of the starch retrogradation, stronger hydrogels (in special at acid conditions), and higher paste clarity. Then, in Chapter 4, the cassava starch was modified by using the combination of both O₃ and DHT treatments. The combination of treatments promoted different modifications concerning the single treatments. Furthermore, the sequence of these treatments influenced many properties of the obtained modified starch. For instance, the samples that were modified with the sequence combination of DHT with O₃ (DHT+O₃) showed the highest degree of oxidation, and a gel more resistant to break than the other hydrogels. On the other side, the starch modified with the opposite sequence (O_3 +DHT) exhibited a degree of oxidation similar to O_3 starch, a molecular size distribution with very small-sized molecules, and capacity to form weaker gels than the native. Finally, the last part of the Thesis (Chapter 5) comprising the General Conclusions and Suggestions for Future Research sections. Therefore, the relevance of the present Thesis is based on the in-depth study of the ozonation process, as well as the novel proposal of combining ozone and DHT treatments, focusing on discuss "green" alternatives for starch modification.

Keywords: starch, modification, ozone (O₃), dry heating (DHT).

RESUMEN Modificación del almidón mediante tecnologías ecológicas

Los almidones son ampliamente utilizados en diferentes industrias y productos. debido a sus diversas propiedades. Sin embargo, los almidones nativos pueden no presentar algunas propiedades particulares requeridas para uso industrial. Para superar limitaciones, la modificación de los almidones es una alternativa para mejorar su desempeño. Sin embargo, la mayoría de los métodos de modificación del almidón utilizados por la industria generan desechos que deben ser tratados adecuadamente para no comprometer el medio ambiente ni los alimentos. Por lo tanto, se necesitan alternativas para satisfacer las necesidades de las industrias sin generar residuos tóxicos. En este contexto, es importante estudiar tecnologías emergentes con atractivo ambiental, también llamadas "tecnologías verdes", como el ozono (O3) y los tratamientos de calentamiento en seco (DHT). Esta Tesis Doctoral se divide en cinco capítulos: El primer capítulo contextualiza este trabajo, que comprende las secciones Introducción y Organización de la tesis, Objetivos y Revisión de la literatura. Luego, se evaluó el proceso de ozonización en dos fuentes de almidón: vuca (Capítulo 2) v arracacha (Capítulo 3). Estos capítulos demostraron que el tratamiento con ozono pudo formar grupos carbonilo y carboxilo, así como causar cambios en la distribución del tamaño molecular (despolimerización), lo que afectó y modificó las propiedades de los almidones de yuca y arracacha. Algunos resultados destacados con aplicación potencial son: reducción de la viscosidad aparente de la pasta, reducción de la retrogradación del almidón, hidrogeles más fuertes (en especial en condiciones ácidas) y mayor claridad de la pasta. Luego, en el Capítulo 4, se modificó el almidón de yuca usando la combinación de tratamientos con O₃ y DHT. La combinación de tratamientos promovió diferentes modificaciones con respecto a los tratamientos individuales. Además, la secuencia de estos tratamientos influyó en muchas propiedades del almidón modificado obtenido. Por ejemplo, las muestras que fueron modificadas con la combinación de secuencia de DHT con O₃ (DHT + O₃) mostraron el mayor grado de oxidación, y un gel más resistente a la rotura que los otros hidrogeles. Por otro lado, el almidón modificado con la secuencia opuesta (O₃ + DHT) exhibió un grado de oxidación similar al almidón O₃, una distribución de tamaño molecular con moléculas de muy pequeño tamaño y capacidad para formar geles más débiles que el nativo. Finalmente, la última parte de la Tesis (Capítulo 5) comprende las secciones de Conclusiones Generales y Sugerencias para Investigaciones Futuras. Por tanto, la relevancia de la presente Tesis se basa en el estudio en profundidad del proceso de ozonización, así como en la novedosa propuesta de combinar tratamientos con ozono y DHT, centrándose en discutir alternativas "verdes" para la modificación del almidón.

Palabras clave: almidón, modificación, ozono (O₃), calentamiento en seco (DHT).

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Chapter 1: Introduction, Thesis Organization, Objectives and Literature review

1. INTRODUCTION AND THESIS ORGANIZATION

Starches are natural ingredients with a great economic relevance once they can be applied in distinct industry sectors, such as follow: chemical, petrochemical, pharmaceutic, cosmetic, food, feed, textile, and paper, among others. However, starches in their native form are limited and exhibit some drawbacks that impairs their further industrial application. Therefore, in order to overcome these drawbacks, different modification processes can be applied in the starch to modify and enhance its functional properties. However, many methods used to modify the starch generate toxic wastes, which can be hazardous to the environment or consumers.

In this context, both ozone (O₃) and dry heating (DHT) technologies are of interest for starch modification. These starch modification techniques are promising for industrial application, because they do not generate toxic wastes for the environmental and for food, being also effective to obtain ingredients with enhanced properties. Consequently, they are considered "green technologies" of processing, resulting in products with "clean label".

In fact, it is essential to understand how those starch modification methods act on native starch granules, if they are effective in modify starch properties and how they can be applicable for industrial use. It is important to highlight that the starch source needs to be considered, in order to attend the industry's reality and demand. Another point that must be considerate is that some starches can be associated with food allergies, such as corn and wheat starches (SICHERER, 1999; BREUNINGER; PIYACHOMKWAN; SRIROTH, 2009).

Therefore, the botanical starch sources chosen for this study were cassava and arracacha, considering some characteristics such as: potential industrial sources, high content of starch, any information about food allergies was obtained (once some starches are related to food allergies, for instance, wheat and maize starches), need to further characterization.

Although some studies were performed with ozonation process in cassava starch, most of them used mild processing conditions, different systems (such as dry process) or did not evaluate the correlation among structure-processing-properties (CHAN et al., 2011; KLEIN et al., 2014; MATTA JUNIOR et al., 2019). The ozonation process was still not evaluated for arracacha starch. Regarding the dry heating technology (DHT), most of the works did not evaluated the pure starch, but so the flours or mixtures of starch with other hydrocolloids (SUN et al., 2013, 2014; CHANDANASREE; GUL; RIAR, 2016; JI et al., 2016; OH; BAE; LEE, 2018; LIU et al., 2019). Furthermore, the combination of O_3 and DHT was not studied yet.

In fact, after gathering all studies about O₃ and DHT, a few questions were raised, such as: a stricter ozonation process condition will be more effective in enhance and change the starch properties? These possible changes will be useful for industrial application? What kind of effects the ozonation process causes in the starch structure (granules and molecules)? How the ozonation process will perform in different starch sources? The combination of DHT with O₃ technology will be more effective in enhancing starch properties? How these combined processes affect starch granules structures and molecules? Will the combination sequence influence the starch structure structures, molecules and properties?

To answer these questions, a further investigation was made with ozonation process to modify cassava and arracacha starches. Furthermore, the combination of DHT with O_3 was evaluated in the cassava starch – third part of the Thesis. Finally, the fourth part (Chapter 5) presents General Conclusions and Suggestions for Future Research.

In this context, this study aims to investigate the effects of ozonation in different starch sources, as also, evaluated the combination of DHT and O_3 treatments in cassava starch. To achieve it, this thesis is divided in four parts and five chapters, as illustrated in Figure 1.



Figure 1. Schematic representation of the Thesis Organization.

The first part (Chapter 1) aims to contextualize this work to the reader; hence, this part comprising the Introduction and Thesis Organization, Objectives, and Literature Review sections. In the second part, the ozonation process was studied in two starch sources: cassava (Chapter 2) and arracacha (Chapter 3), which were published in the journals "Ozone: Science and Engineering" and "Food Hydrocolloids", respectively. The effect of this technology was evaluated concerning the starch structure (both granule and molecules) and properties, focusing on the correlation among structure-processing-properties. In Chapter 4, the combination of DHT and O₃ treatments were evaluated for cassava starch modification, which was published in the journal of Biological Macromolecules".

the Thesis, Chapter 5 describes the General Conclusions and Suggestions for Future Research sections.

2. GENERAL OBJECTIVES

The main objective of this Thesis was to enhance the native starch properties using the ozone technology alone or in combination with the dry heating treatment (DHT). In this context, some specific objectives were intended:

- Evaluate the effect of ozone processing on cassava and arracacha starch structures (molecules and granules) and properties, promoting correlations among them;
- b. Use the combination of ozone and dry heating treatments (in different sequences) to modify cassava starch, evaluating their effects on starch molecules and properties, as also provide an explanation to correlate the hydrogels texture behavior with molecular properties (size distribution and functional groups).

3. LITERATURE REVIEW

3.1. Starch

Starch is the main compound in plants that is used as storage carbohydrate and it is a complex material, mainly formed by two polymers of D-glucose, named amylose and amylopectin (Figure 2), which represent approximately 98-99% of the dry weight (TESTER; KARKALAS; QI, 2004; COPELAND et al., 2009). Both type of molecules are made of α -1,4-linked glucan chains, being amylopectin also formed by ramifications connected through α -1,6-branch points, (PFISTER; ZEEMAN, 2016). The proportion of these two polymers vary according to harvest period and botanical origin.



Figure 2. Amylose and the amylopectin molecules.

Amylose is typically linear macromolecule, being composed by α -Dglucopyranose residues linked together by α -1,4-bonds, but some molecules are slightly branched by α -1,6-linkages (BULÉON et al., 1998; TESTER; KARKALAS; QI, 2004; LIU, 2005). Amylose has a molecular weight of about 10⁵-10⁶ g/mol, the degree of polymerization (DP) of 1,000-10,000 glucose units (TESTER; KARKALAS; QI, 2004; COPELAND et al., 2009; JANE, 2009). Amylose presents a low degree of branching, which results in a tendency to form insoluble semi-crystalline aggregates (COPELAND et al., 2009). This compound is much prone to a intermolecular association of amyloses (retrogradation process), and can form tough gels and strong films (PÉREZ; BERTOFT, 2010; WITCZAK et al., 2016). Amylopectin is highly branched and it is formed by short 1,4-linked α -Dglucopyranosyl units, which are interlinked by α -1,6-linkages (BULÉON et al., 1998; TESTER; KARKALAS; QI, 2004). The amylopectin molecule is much larger than amylose, presenting a molecular weight of 10⁷-10⁹ g/ mol and a DP that may overtake one million (TESTER; KARKALAS; QI, 2004; COPELAND et al., 2009; JANE, 2009). Different from amylose, amylopectin is capable of disperse in water and retrogrades much slower, which originates soft gels and weak films (PÉREZ; BERTOFT, 2010; WITCZAK et al., 2016).

The amylose and amylopectin content are responsible for many functional properties of starch. including gelatinization, pasting, gelling, and retrogradation/syneresis (AI; JANE, 2018). However, the starch complexity can be highlighting by observing how those molecules are naturally present inside plant cells: both amylose and amylopectin are naturally arranged in granules, formed by amorphous and crystalline regions. The starch granules may vary in functional properties, size and morphology according to its botanical source. The diameter of starch granules ranges from 1 to 100 µm (LINDEBOOM; CHANG; TYLER, 2004; PÉREZ; BERTOFT, 2010; VAMADEVAN; BERTOFT, 2015; PFISTER; ZEEMAN, 2016).

Generally, native starch granules are insoluble in cold water. But, when the starch, is heated in the presence of a sufficient amount of water, the granules absorb water and swell irreversibly. The amylose leaches out of the granules and solubilize. As the temperature and water absorption increase, the granules rupture with the disordering of the organization of the chains (ZHU, 2015). This phenomenon is known as gelatinization. If the concentration of the starch in the paste is high enough (usually higher than ~ 5-6%, depending on the starch source), intermolecular interactions involving amylose and amylopectin molecules occur after cooling the paste, resulting in the gel formation (MORRIS, 1990; COPELAND et al., 2009; ZHU, 2015; CASTANHA et al., 2020).

Due to starch capacity of absorb water and form gels, among other properties, being also easily to be obtained, cheap, biodegradable and abundant, it is a component with a high industrial and academic interest. In fact, starches are commonly used in different industries, such as food, feed, chemical, petrochemical, textile, pharmaceutical, cosmetics, paints, etc. Figure 3 presents some examples of products that uses starch as an ingredient for their production.



Figure 3. Examples of some products that uses starch as an ingredient.

3.2. Different starch botanical sources

3.2.1. Cassava starch

Cassava (*Manihot esculenta* Crantz) (Figure 4), also known as tapioca, manioc, or yuca, is a perennial plant with tuberous roots and it is considered an important food crop with a considerable nutritional value. It is a native plant from South America, being widely cultivated in tropical and subtropical regions of Asia, Africa, and Latin America (TONUKARI, 2004; ZHU, 2015; SÁNCHEZ et al., 2017).



Figure 4. Cassava roots.

There are some common industrial applications for the cassava root, such as starch, flour, chips, and pellets (TAIWO, 2006; FALADE; AKINGBALA, 2010). However, the main product extract from cassava roots is the starch which has a relevant industrial application.

The starch content of cassava roots is about 65-91% (on dry basis), and depending on the variety, the content of amylose range from 14 to 30%, as for amylopectin, is around 70 to 75% (ZHU, 2015; MOORTHY; SAJEEV; ANISH, 2018; TAPPIBAN et al., 2019). The shape of cassava starch granules is described as oval, truncated, and rounded; regarding its surface, in general, is smooth (ZHU, 2015).

Cassava starch has different physicochemical properties and functional characteristics that contribute to the texture properties of many foods. Besides improving the texture of different food products, the cassava starch can also improve its appearance and properties, by presenting a paste with elevated clarity and apparent viscosity, as well as with low gelatinization temperature and low retrogradation tendency (DÍAZ et al., 2018).

3.2.2. Arracacha starch

Arracacha (*Arracacia xanthorrhiza*, Bancroft) (Figure 5), also called Peruvian carrot, Peruvian parsnip, White carrot, and others, is a tuberous root originally from the Andean South America and its productions concentrate in South America (mainly in Brazil, Colombia, Venezuela, Ecuador, Peru, and Bolivia) (ALBANO; FRANCO; TELIS, 2014; LONDOÑO-RESTREPO et al., 2018; SANTOS et al., 2018).



Figure 5. Arracacha roots.

In South-America, arracacha is frequently used in soups, bread, chips, and it is an important source of starch (ROCHA et al., 2011; LONDOÑO-RESTREPO et al., 2018). However, arracacha roots present low commercialization, industrialization, and consumption, which make it an "underutilized" crop (CASTANHA et al., 2018). In order to enlarge arracacha roots use, a good alternative would be starch extraction, since the roots present a high content of starch and according to some works, it has a great potential for industrial application (LEONEL et al., 2016; CASTANHA et al., 2018; LONDOÑO-RESTREPO et al., 2018).

Arracacha presents a high content of starch, as presented in some works it ranges from 20 to 80% (ALBANO; FRANCO; TELIS, 2014; CASTANHA et al., 2018; LONDOÑO-RESTREPO et al., 2018; SANTOS et al., 2018). The amylose content of arracacha, reported in different studies, is about 15 to 40% (ROCHA et al., 2011; ALBANO; FRANCO; TELIS, 2014; MORAES; BRANZANI; FRANCO, 2014; CASTANHA et al., 2018; LONDOÑO-RESTREPO et al., 2018). The arracacha granule starch, in general, presents a round, small and polygonal shape, some granule exhibits cracks and fissures on their surfaces, presenting a "Lego-like" arrangement (CASTANHA et al., 2018; LONDOÑO-RESTREPO et al., 2018).

Arracacha starch has fragile granules (due to its weak crystalline structure), and as for its properties, they presented a low gelatinization temperature, low enthalpy of gelatinization, low retrogradation, and syneresis (ROCHA et al., 2011; MORAES; BRANZANI; FRANCO, 2014; CASTANHA et al., 2018; LONDOÑO-RESTREPO et al., 2018).

3.3. Starch modification

Starch is widely used for industrial applications due to its low cost, availability, and broad range of functional properties that permit its use in food and non-food products. However native starch may not present some particular properties that are required for industrial use. Therefore, to overcome the shortcomings of native starches, starch modification is an alternative to improve starch properties for industrial applications. The main types of starch modification are physical, enzymatical, and chemical (KAUR et al., 2012).

Although many technologies are available, we highlight the rising trend for using "green technologies" for starch modification, i.e., technologies whose environmental impact is minimal. In this scenario, we highlight the ozone (O₃) as a chemical technology and dry heating treatment (DHT) as a physical technology.

3.3.1. Ozone technology and its use in starch modification

Ozone (O₃), a gas with a pungent odor, found in our atmosphere, is considered a powerful oxidizing agent with a high oxidation-reduction potential of 2.07 V (MAHAPATRA; MUTHUKUMARAPPAN; JULSON, 2005; PANDISELVAM et al., 2019). At normal temperatures and pressures, ozone is an unstable gas and partially soluble in water (KHADRE; YOUSEF; KIM, 2001; MAHAPATRA; MUTHUKUMARAPPAN; JULSON, 2005). Ozone decomposes rapidly at room temperature and does not accumulate considerably without continual ozone generation (GUZEL-SEYDIM; GREENE; SEYDIM, 2004).

The ozone's solubility in water is higher in cold water, however, it decomposes rapidly at higher water temperatures (Table 1) (MAHAPATRA; MUTHUKUMARAPPAN; JULSON, 2005).

| Temperature (°C) | Solubility (L ozone/ L water) |
|------------------|-------------------------------|
| 0 | 0.640 |
| 15 | 0.456 |
| 27 | 0.270 |
| 40 | 0.112 |
| 60 | 0.000 |

Table 1. Temperature and solubility of ozone in water.

Source: Mahapatra, Muthukumarappan e Julson (2005).

For years the ozonation process has been used as a sanitizer in the food industry. However, due to ozone characteristics, it can also be used as a starch modification technology. The process of ozone formation can be done by various techniques, including corona discharge, ultraviolet radiation, electrolysis, and chemical radiation (HANDARINI et al., 2020). In fact, the ozone process is an attractive chemical technology of starch modification because it decomposes into oxygen, leaving no toxic residues in food or the environment, is considered a "green"/ "environmentally friendly" technology (CHAN; BHAT; KARIM, 2009a; VANIER et al., 2017). Literature reports two main systems used to modify starch by ozone technology: liquid system and powder system (MANIGLIA et al., 2020b). In the liquid system, the starch is suspended in distilled water, whereas in the powder system, the starch is placed in an empty vessel. Furthermore, ozone technology is a very versatile and cheap modification process, once it is possible to use industrial oxygen or atmospheric air to produce O_3 and applied it in the modification system. The schematic representation of ozone technology is displayed in Figure 6.



Figure 6. Schematic representation of ozone technology, illustrating two different reactors, where Reactor 1 is used for liquid systems and Reactor 2 is used for powder systems.

In fact, ozone used as a starch modification technology had been applied in different starch sources, most of the works were performed in mild ozonation conditions (AN; KING, 2009; CHAN; BHAT; KARIM, 2009b; CHAN et al., 2011, 2012; OLADEBEYE et al., 2013; KLEIN et al., 2014; CASTANHA; MATTA JUNIOR; AUGUSTO, 2017; CASTANHA et al., 2019; MATTA JUNIOR et al., 2019). According to Handarini et al. (2020) and Maniglia et al. (2020b), the main reactions that occur during the ozonation process of starch are the oxidation of the hydroxyl groups, forming carbonyl and carboxyl groups (Figure 7); and reduction of the size of the molecules (depolymerization). Thus, due to these reactions, there are changes in the structure of starch that will reflect in its properties. However, it is necessary for a further investigation on its effects on starch molecular structures and also how the modified starch will be performed in the industry's products.



Figure 7. Oxidation of hydroxyl groups, and formation of carbonyl and/ or carboxyl groups. Source: adapted from Castanha (2016).

3.3.2. Dry heating technology

The dry heating technology (DHT) involves the modification of the physicochemical characteristics of native starch, such as, swelling power, solubility, color, and viscosity, which enhances its functional characteristics that could be used to tailor starch for food applications (CHANDANASREE; GUL; RIAR, 2016). Recently, this modification method has received great attention due to its simplicity, safety, and for being considered a "green technology"; (OH; BAE; LEE, 2018; MANIGLIA et al., 2020b).

DHT treatment, generally, consists of heating the starch with a low moisture level (7 – 13%) at high temperatures, which ranges from 110 to 200°C (MANIGLIA et al., 2020b). Moreover, according to Maniglia et al. (2020b), the DHT oxidizes the hydroxyl groups, forming carbonyl groups, and reduces the size of the molecules. Besides that, the DHT also able to produce strong gels, that is interesting for 3D food printing application (MANIGLIA et al., 2020a). Figure 8 presents a schematic representation of DHT technology.



Figure 8. Schematic representation of DHT technology.

Different studies have been carried out with this modification process, however, most of them are concentrated on the combined effect of DHT with the addition of compounds in the starch (e.g.: hydrocolloids) (SUN et al., 2013, 2014; JI et al., 2016; OH; BAE; LEE, 2018; LIU et al., 2019). Therefore, would be interesting to evaluate DHT process effects in pure starch and/or also use this technique with other modifications methods.

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Chapter 2: Ozone processing of cassava starch*

LIMA, D. C.; CASTANHA, N.; MANIGLIA, B. C.; MATTA JUNIOR, M. D.; LA FUENTE, C. I. A.; AUGUSTO, P. E. D. Ozone Processing of Cassava Starch. **Ozone: Science & Engineering**, p. 1–18, 2020.

*A patent application was filed regarding part of obtained results.

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ABSTRACT

This work evaluated the ozonation of cassava starch, correlating the process conditions with starch structure and properties. The starch structure was evaluated considering both molecules and granules. Ozonation resulted in an increased number of carbonyl and carboxyl groups with increasing ozone consumption. Moreover, a reduction of large and branched molecules was observed, with the consequent increase of smaller sized molecules. The structural changes significantly influenced the pasting properties, gel texture, water absorption and solubility indexes. As the main result, ozonation improved the hydrogels' texture. The 145.3 mg O₃ g_{starch}⁻¹ sample produced gels ~280% stronger than the native starch. Moreover, the ozonated starch hydrogels maintained their consistency even at acidic conditions – on the contrary to native starch. Finally, the ozonated starch pastes were clearer than native ones. Therefore, ozone processing gives new possibilities for cassava starch application.

Keywords: Ozone; ozonation process; green technologies; starch modification; cassava starch.

1. INTRODUCTION

Starches are natural ingredients relevant from an economic point of view since they are widely used in different industries, such as food (human and animal), pharmaceutic, cosmetic, chemical, petrochemical, paper, and textile, among others.

Starch granules, molecules, and trace elements may vary in form and functionality, according to its botanical origin. This variability provides starches with different physicochemical properties (COPELAND et al., 2009). However, starches in their native form present limited uses for industrial application, such as poor shear and freeze-thaw stability, high degree of retrogradation, water insolubility, unstable pastes and gels (ASHOGBON; AKINTAYO, 2014; VANIER et al., 2017; ZIA-UD-DIN; XIONG; FEI, 2017; DAR et al., 2018). Therefore, to overcome those limitations, different starch modification technologies have been used as an alternative to increasing their functionality for different applications.

Chemical, enzymatic and physical processes are used for starch modification. Chemical modification through acetylation, oxidation, cross-linking, hydroxypropylation and etherification are the most commonly applied (VANIER et al., 2017). However, by using chemical agents, different residues are produced, which can be unsafe for consumers, employees, and/or environment, or, at least, demand treatment and limit "clean labels". Ozone (O₃) is an attractive chemical technology for starch modification because it decomposes into oxygen, leaving no toxic residues in food or environment, and is considered a "green" and "environmentally friendly" technology with "clean label" (PANDISELVAM et al., 2017, 2019). Starch ozonation is further than a simple oxidation process, once this technology is able to promote important changes in both molecular size and chemical properties, leading to new properties and applications (CASTANHA; MATTA JUNIOR; AUGUSTO, 2017; CASTANHA et al., 2019).

Although starch modification using ozone was already studied for different sources, a relevant lack of knowledge is still observed.

For instance, some of the evaluated starches do not attend the industry's reality and demand, due to limited production, such as cocoyam, yam, lima bean, pigeon peas, jack bean and sago (CHAN; BHAT; KARIM, 2009; CHAN et al., 2011, 2012; OLADEBEYE et al., 2013, 2018). Further, some starch sources have their use impaired, once they are associated with food allergies (SICHERER, 1999; BREUNINGER; PIYACHOMKWAN; SRIROTH, 2009), such as corn and wheat starches (Çatal and İbanoğlu 2012a; 2012b; 2014; Chan et al. 2012; Chan, Bhat, and Karim 2009; Chan et al. 2011). Moreover, some works only applied mild ozonation conditions and did not observe significant changes (AN; KING, 2009; ÇATAL; İBANOĞLU, 2013).

On the other hand, some promising sources are underexplored, being necessary further studies to better understand the process and propose alternatives for industrial applications.

For instance, cassava is a promising starch source. Cassava (*Manihot esculenta* Crantz) shrub presents high tolerance to drought and harsh climatic conditions and around-the-year availability, and its roots are rich in starch (ZHU, 2015). Cassava starch has distinct functional characteristics and physicochemical properties such as presenting low gelatinization temperature and low retrogradation tendency (MOORTHY; SAJEEV; ANISH, 2018). However, industrial applications of cassava starch may present limitations, in special its weak gel.

In order to overcome these limitations and taking this promising crop, modification strategies have been evaluated. In fact, some studies were conducted with the ozonation process to modify cassava starches, although with limitations. 40

Chan, Bhat, and Karim (2009), and Chan et al. (2011) used a dry process (i.e., submitting the starch powder to a gas stream containing ozone) to ozonate cassava starch (and also corn and sago starches). However, the dry process is not as effective as an aqueous process. In fact, Chan et al. (2011) could not even measure the ozonated cassava starch gels, since they presented weak formation. Furthermore, the dry process actual industrial application is limited and it is even dangerous due to possible explosions. Other studies have used interesting approaches to describe the process, but with industrial limitation. Klein et al. (2014) studied the effects of the ozonation technology in aqueous suspension of cassava starch at different pHs (3.5, 6.5, and 9.5). Although their approach is interesting to understand some aspects of the reaction, the industrial process at different pHs is difficult and limit the possible "clean label" of ozonation, since it is necessary the use of chemical agents (HCI and NaOH) to vary the pHs (in special considering the food and pharmaceutical industries). Moreover, their process conditions were mild and the authors stated that most of the changes were influenced by the pH of the reaction and the ozonation process had a minor effect on the samples. Finally, other authors focused on specific applications, but without evaluating further aspects. Matta Junior et al. (2019) studied ozone technology as an alternative to the fermentation process in cassava starch, focusing on oven-expansion. The work was conducted under mild process conditions, as well as few characterization analyses were performed.

Therefore, there is still a lack of knowledge and in-depth discussion considering the ozonation process of cassava starch. In special, the characterization of processstructure-properties relationship considering industrial conditions is still poorly explored. Moreover, no studies have been conducted to evaluate the potential use of the ozonation process to improve the gel texture and paste clarity of cassava starch.

Consequently, this work studied structural changes in cassava starch modified by ozone, correlating them with physicochemical and functional properties, as well as investigating possible applications in different industries.

2. MATERIAL AND METHODS

2.1. Raw material and ozonation process

The native cassava starch used in this study was a regular commercial product, with a purity of ~99%, and kindly donated by Cargill Agrícola S.A. (Amilogill 1500; Campinas, Brazil).

The ozonation process was carried in a laboratorial scale, using the same system described by Castanha, Matta Junior, and Augusto (2017). Ozone was produced in an ozone generator unit (Ozone & Life, O&G Model RM 3.0, São José dos Campos, Brazil) by the coronal-discharge method from industrial oxygen (95% purity). 1.5 L of a starch dispersion in distillate water (10% m/m in dry basis) was placed in a glass reactor (2 L capacity, 56 cm of height, 6 cm of internal diameter) and processed for 15, 30 and 60 minutes, being constantly stirred with a magnetic stirrer. The gas flowing into the reactor was maintained constant at 1 L min⁻¹, and the inlet ozone concentration was kept at ~43.5 mg O₃ L⁻¹. Ozone concentration entering and leaving the reactor was monitored using an ozone monitor (2B Technologies, Model 106-H, Boulder, USA). The ozone concentration in each stream (reactor inlet and outlet), with the information collected from this ozone monitor, is shown in section 3.1. The gas stream was bubbled into the starch suspension. The system, thus, is a multiphase problem with three different phases and physical states: gas (O₂ + O₃), liquid (water), and solid (starch granules).

The native starch (10% m/m in dry basis) was also suspended in 1.5 L of distilled water - without being ozonated – and constantly stirred with a magnetic stirrer. This starch was used as control (being named as "0 mg O₃ $g_{\text{starch}^{-1}}$ ").

Then, the starch suspensions - native and ozonated samples - were maintained at rest for one hour in order to settle the starch granules, and the supernatant was discarded. The starch was dried in an air circulation oven at 35 °C until ~12% of moisture content (on wet basis). The dried samples were then milled in a mortar, sieved in a 60-mesh sieve (250 μ m) and kept closed in glass flasks for further analysis.

2.2. Starch molecular characterization

2.2.1. pH

The pH was determined by a calibrated potentiometer (Tecnal, TEC-5 mode, Piracicaba, Brazil). Five grams of starch sample was suspended in 50 mL of distilled water, being constantly stirred with a magnetic stirrer (ADOLFO LUTZ INSTITUTE, 2008).

2.2.2. Carbonyl and carboxyl groups contents

The carboxyl groups content was determined according to the procedure of Chattopadhyay, Singhal, and Kulkarni (1997), with modifications. 3 g of starch was dispersed in 25 mL of 0.1 M HCl and stirred with a magnetic stirrer for 30 minutes. The samples were then vacuum-filtered using a qualitative filter paper (205 µm), and washed with 400 mL of distilled water. The obtained starch was transferred into a 600 mL beaker and it was suspended in 300 mL of distilled water, then brought to a boiling water bath with continuous manual stirring for 15 minutes to ensure gelatinization. The hot gelatinized starch was then titrated with 0.01 M NaOH using an automatic titrator (Plus Titrino 848, Metrohm, Herisau, Switzerland) until pH 8.3. An unmodified starch was used to perform a blank test. The result was expressed in the quantity of carboxyl groups per 100 glucose units (COOH/100GU). Carboxyl groups content was calculated using the following Equation (1) (where Vs is the volume of NaOH required for the ozonated samples, Vb is the volume of NaOH used in the blank, M is the molarity of NaOH and W is the sample mass in dry basis):

COOH/100GU = [(Vs - Vb) M 0.045 100]/W(1)

The carbonyl groups content was determined as the methodology described by Smith (1967). The starch (4 g, on dry basis) was suspended in 100 mL of distilled water. The suspension was gelatinized in a boiling water bath for 20 minutes and the obtained gel was cooled to 40 °C, adjusted to pH 3.2 with HCl 0.1 M. 15 mL of hydroxylamine solution (made with 10 g of hydroxylamine hydrochloride, 40 mL of 0.5 M NaOH solution and distilled water until the solution reach 200 mL) were added to the sample. Then the samples were placed in a 40 °C water bath with stirring for 4 h. Thereafter, the excess of hydroxylamine was titrated with 0.1 HCl, using an automatic titrator (Metrohm, 848 Tritino plus, Herisau, Switzerland), until pH 3.2. A blank, without sample, was prepared with all reagents. The result was expressed in the quantity of carbonyl groups per 100 glucose units (CO/100GU). Carbonyl groups content was calculated using the following Equation (2) (where Vb is the volume of HCl used in the blank, Vs is the volume of HCl required for the sample, M is the molarity of HCl and W is the sample mass in dry basis):

2.2.3. Gel permeation chromatography

The molecular size distribution profile of the starch molecules was evaluated by gel permeation chromatography (GPC), according to the methodology of Song and Jane (2000), with some modifications. A GE XK 26/70 column (2.6 cm diameter and 70 cm high) packed with Sepharose CL-2B gel (Sigma, Stockholm, Sweden) was used. Starch samples (0.1 g, on dry basis) was were dispersed in 10 mL of dimethylsulfoxide (DSMSO; 90%, Labsynth, Diadema, Brazil) and heated in a boiling water bath for 1 h and kept at 25 °C for 16 h under constant stirring. An aliquot of 3 mL was then mixed with 10 mL of absolute ethanol. Thereafter, the solution was centrifuged for 30 minutes at 3000 x g. The supernatant was discarded, and the precipitated starch was dissolved in 9 mL of boiling distilled water, then placed in a bath of boiling water for 30 minutes. 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 fractions of 4 mL. The samples were evaluated by the blue value method (JULIANO, 1971): I₂ (0.1 g) and the KI (1.0 g) where dissolved in 100 mL of acetate buffer 0.2 M at pH 5.0 to prepared an I₂/KI solution. An aliquot of 100 µL of this solution was added in the fractions, then the samples were evaluated using a spectrophotometer at a wavelength of 620 nm (spectrometer Femto, Model 600S, São Paulo, Brazil).

2.2.4. Apparent amylose content

The apparent amylose content was determined according to the ISO methodology (1987). A standard curve was made by using standard amylopectin (A8515) and amylose (A0512 type III), both from Sigma-Aldrich Pty Ltd. (Casle Hill, Australia). Starch samples (0.1 g, on dry basis) were dispersed in 1 mL of ethanol 95% and 9 mL of NaOH 1 mol L⁻¹. Then, the samples were gelatinized in a boiling water bath for 10 minutes and cooled at room temperature. An aliquot of 18 mL of gelatinized sample was mixed with 2 mL of NaOH 0.09 mol L⁻¹. Thereafter, an aliquot of 5 mL of the solution was placed in a volumetric flask of 100 mL containing 1 mL of acetic acid 1 mol L⁻¹. The volumetric flask was stirred, then it was added 2 mL of iodine solution (0.2% I₂, 2% KI, in distilled water) and the flask was filled with distilled water. After 20

minutes, the samples were evaluated in a spectrophotometer at a wavelength of 620 nm (spectrometer Femto, Model 600S, São Paulo, Brazil).

2.3. Starch granule characterization

2.3.1. Granule morphology

The starch granules morphology was evaluated by scanning electronic and light microscopies.

The scanning electronic microscopy (SEM) was carried out using a LEO 435 VP, Leo Electron Microscopy Ltd. (Cambridge, England) operating at an acceleration voltage of 20 kV. To prepare the samples, a double sticky carbon tape was fixed on a circular aluminum stub, and dry starch was sprinkled on the tape. A brush was used to ensure a uniform layer of powder particles. The stubs were coated with gold (~30 nm) and then evaluated in the microscope.

To observe the starch granules shape of surface and edges, a light microscope was also used (model L1000, Bioval, Curitiba, Brazil), with a 20 W halogen lamp. The samples (0.1 g) were dispersed in 10 mL of distillate water with 50 μ L of Lugol. Then, using a Pasteur pipette, a drop of this solution was placed on a glass slide covered by a glass coverslip. The magnification used to obtain the images was of 400x.

2.3.2. Particle size distribution

The particle size distribution was performed using a Laser Analyser (Partica LA-950V2 Laser Particle Size Analyser HORIBA, Kyoto, Japan) and to evaluate the data it was used the LA-950 software for Windows (HORIBA, Kyoto, Japan). The samples were dispersed in ethanol (99.5%). The volume-based mean diameter (D [4,3]) and the area-based mean diameter (D [3,2]) were determined according to the equations below, where n_i is the number of particles of diameter d_i :

$$D[4,3] = \frac{\sum_{i} n_{i} d_{i}^{4}}{\sum_{i} n_{i} d_{i}^{3}}$$

$$D[3,2] = \frac{\sum_{i} n_{i} d_{i}^{3}}{\sum_{i} n_{i} d_{i}^{2}}$$
(3)

The D [4,3] is influenced by large particles, while the D [3,2] is influenced by smaller ones (LOPEZ-SANCHEZ et al., 2011).

2.3.3. Granule relative crystallinity

The starch samples were maintained in a desiccator containing saturated BaCl₂ (25 °C, aw = 0.90) for 10 days to equilibrate the moisture content. Then, to determine the X-ray diffraction patterns, the samples were subjected to an X-ray diffractometer (Rigaku Miniflex 600 RXD, Tokyo, Japan) with Cu radiation, at an angle 20 ranging from 3 to 50°. The scan rate was 2° min⁻¹, with a voltage of 30 kV and amperage of 15 mA. The relative crystallinity was determined as described by Nara and Komiya (1983), using the Origin software version 9.6 (Microcal Inc., Northampton, USA). The graphics were plotted between 20 angles from 3 to 30° and the curves were smoothed using the Origin software tools.

2.4. Starch properties and possible applications

2.4.1. Pasting properties

The determination of the pasting properties was made by using RVA-4 (Newport Scientific Pvt. Ltd., Warriewood, Australia, with the Thermocline for Windows software, version 3.0). A suspension of 3 g of starch (corrected to 14% moisture in wet basis) in 25 g of distilled water was analyzed according to a programmed heating, retention and cooling cycle under constant shear: The suspensions were first held at 50 °C for 1 min, then heated to 95 °C, being then kept at 95 °C for 5 min, followed by cooling to 50 °C, and finally holding it at 50 °C for 2 min.

2.4.2. Gel strength

In order to measure the gel strength, starch hydrogels were prepared using two approaches: Firstly, the gels were prepared using 10.7% of starch in water, as normally. However, acid foods are an important application for starch, whose consistency is impaired by acid conditions. Therefore, in the second approach, the gels were prepared using 10.7% starch in citric acid pH 3 (0.08% of citric acid in distilled water). The pH values of the suspensions, before gelatinization, are presented in Supplementary Table 1.

| pH (Water) | pH (Citric Acid) |
|---------------|--|
| 5.86 ± 0.08 a | 3.00 ± 0.02 a |
| 3.99 ± 0.08 b | 3.02 ± 0.00 a |
| 3.82 ± 0.03 c | 3.02 ± 0.00 a |
| 3.63 ± 0.02 d | 2.98 ± 0.05 a |
| | pH (Water) 5.86 ± 0.08 a 3.99 ± 0.08 b 3.82 ± 0.03 c 3.63 ± 0.02 d |

Supplementary Table 1. pH of starch suspensions before gelatinization.

The starch samples were gelatinized under vigorous mechanical agitation, using a mechanical stirrer (250 rpm), in a boiling water bath for 15 min. The obtained gels were stored at 5 °C for 24 h in a 40 x 20 mm (diameter x height) plastic cup in a desiccator with water at the bottom (to ensure uniform moisture). The gel strength, or firmness of the starch hydrogels, was determined by a puncture assay using a Texture Analyzer (TA.XT Plus, Stable Micro Systems Ltd., Surrey, UK) with a load cell of 50 kgf (490.3 N). It was used a cylindrical probe (P/0.5R, 12.7 mm of diameter) to compress the samples until the distance of 10 mm at 1 mm s⁻¹.

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

The water absorption and solubility indexes were performed as described by Anderson, Conway and Peplinski (1970), with some modifications. Starch samples (0.5 g, on dry basis) were dispersed in 6 mL of distilled water in pre-weighed centrifuge tubes. The tubes were placed in a shaking thermal bath for 30 min at different temperatures (60, 65, 70, 75, 80, 85, 90, and 95 °C), and then centrifuged at 3000 x g for 10 min. The supernatant was placed in a pre-weighed glass Petri dish, dried at 105 °C for 16 h, then cooled and weighed (DS). The precipitated retained in the tube was also weighed (PR). The WAI (Eq. (5)) and WSI (Eq. (6)) were calculated considering MS (mass of the sample, on dry basis).

WSI (%) = (DS/MS)·100

(6)

2.4.4. Paste Clarity

Paste clarity was determined as described by Aplevicz and Demiate (2007), but also evaluating it over the storage time. Samples were prepared mixing 50 mL of

distilled water and 0.5 g of starch in test tubes with a screw cap. Then, the tubes were kept in a thermal bath with boiling water for 30 min and stirred individually 5 times every 5 minutes. After the thermal bath, the samples were stored at 5 °C for 20 days. During this period, the paste clarity measurements were carried out in the following days: 0 (considered the day that the paste was made), 1, 3, 5, 8, 12, 16, and 20. The paste clarity of the samples was evaluated by transmittance measurement (T%) in a spectrometer at 650 nm wavelength (spectrometer Femto, Model 600S, São Paulo, Brazil).

2.5. Experimental design and statistical analysis

The experimental design was completely randomized. Each processing time was made in triplicate and the samples were analyzed in duplicate. Data were assessed by the analysis of variance (ANOVA) and comparisons of averages for treatments were made using Tukey's test using the software Minitab version 18 (Minitab, LLC., Pennsylvania, USA).

3. RESULTS AND DISCUSSION

3.1. Ozone-starch reaction

The inlet and outlet ozone (O_3) concentration in the reactor during starch processing are shown on Figure 1, as well as the total O_3 consumption during the reaction. The concentrations, in Figure 1-B, are expressed in relation to the starch mass into the reactor.



Figure 1. (A) Inlet and outlet ozone concentration in the reactor; and (B) ozone consumption during the starch modification process.

The inlet O_3 concentration was ~43.5 mg L⁻¹ and the outlet O_3 concentration quickly increase until reaching a stationary concentration of ~30.6 mg L⁻¹ (Figure 1-A). The outlet concentration is stationary, after a few minutes, possibly due to the O_3 reaction with the starch suspension that are constant: this demonstrates the ozone is in excess in our system, which is important to study and understand the reaction. Figure 1-B demonstrates the total O_3 consumed during the ozonation process increased almost linearly with time.

Nevertheless, it is worth mention the system must be optimized for industrial purposes, considering different aspects. First, the system presents three different phases and physical states - gas ($O_2 + O_3$), liquid (water), and solid (starch) – which influence the reaction of the ozone with starch. Second, starch granules (solid phase) are insoluble in water (liquid phase) at room temperatures. Therefore, in this process conditions, starch makes a suspension, not a solution in water. Third, the O_2 and O_3 (gas phase) is dispersed in the starch suspension through small bubbles, and only part of them dissolve in the water: the amount of dissolved gas is a function of many different parameters, such as bubbles dimensions and retention time, the contact between ozone and starch, the geometry and dimensions of the reactor and agitation system, the suspension consistency (which is a function of both starch concentration and temperature), etc. In this way, the ozone processing of starch is more complex than a direct chemical reaction.

Even though, this approach allowed us to study and understand the ozonation process of cassava starch, observing significant changes in starch molecules - which resulted in modification of starch properties – as detailed in the following sections.

Finally, this work presents the ozonation process through processing time at the conditions described in the "material and methods" section. Even so, considering Figure 1-B, a correlation between ozone processing time and ozone consumption (which represents the amount of O_3 that reacted with the starch suspension), and it was calculated as proposed by Leeuwen (2015). It was observed that: 0 min = 0 mg $O_3 g_{starch}^{-1}$; 15 min = 78.9 mg $O_3 g_{starch}^{-1}$; 30 min = 145.3 mg $O_3 g_{starch}^{-1}$; and 60 min = 270.7 mg $O_3 g_{starch}^{-1}$.

3.2. Starch molecular characterization: pH, carbonyl and carboxyl content, molecular size distribution and amylose content

In Figure 2 four different aspects of native and ozonated cassava starch structure are shown: carbonyl and carboxyl contents (Figure 2-A), the pH of the starch suspensions (Figure 2-B), the size distribution of starch molecules (Figure 2-C) and the apparent amylose content (Figure 2-D).



Figure 2. (A) Carbonyl and carboxyl content, (B) pH values, (C) molecular size distribution profile (blue value method) and (D) apparent amylose content of native and ozonated cassava starch. The dots are the mean values and the red vertical bars are the standard deviation. Variations followed by the same small letters do not differ significantly (p < 0.05).

The carbonyl and carboxyl groups content increased with an increase in O_3 consumption, as presented in Figure 2-A. The presence of carboxylic groups increases the acidity of starch, reducing the pH of the suspension (Figure 2-B).

During the ozonation process, the oxidation of the hydroxyl groups was observed, forming carboxyl and carbonyl groups, probably at the carbons 2, 3 and 6, as discussed by Wurzburg (1986). Usually, in conventional oxidation processes, it is assumed that the carboxyl group content would be higher than the carbonyl group, since carbonyl is an intermediate compound in the reaction of hydroxyl to carboxyl. However, the carbonyl content was higher than the carboxyl content (Figure 2-A), which highlights the ozonation as a special process of oxidation. It is important to highlight that ozone reacts not only with the starch hydroxyl groups, but also ozone decomposes in water, forming secondary oxidants, such as hydroxyl radical (MASTEN; DAVIES, 1994; KHADRE; YOUSEF; KIM, 2001). Each specie has its reactiveness, being the reaction complex. Moreover, the ozone reactivity is high enough to promote both oxidations simultaneously, unlike in other oxidative processes, whereby carbonyl formation is observed first and then carboxyl formation occurs.

These results are similar with presented in other works (Chan, Bhat, and Karim 2009; Chan et al. 2012; Oladebeye et al. 2013; Castanha, Matta Junior, and Augusto 2017; Oladebeye et al. 2018). However, it deviates from some authors, such as Matta Junior et al. (2019), while studying ozonated cassava starch to improve ovenexpansion properties. The authors observed a carboxyl content higher than the carbonyl content, which can be attributed to the mild processing conditions. Klein et al. (2014), evaluating the effects of the ozonation process at different pH, noticing lower values of carbonyl and carboxyl. The highest values of these compounds were found at pH of 9.5 (0.011 and 0.028%, respectively), and are lower than in this study. The authors attributed their low content of carbonyl and carboxyl to the high starch concentration in the water during the ozonation (200 g of starch/300 mL of distilled water), which impaired the effectiveness of the ozonation process. Cahyana et al. (2018), studying ozonation of banana flour and starch, noticed a higher amount of carboxyl in the starch (~0.0235%) than in the flour (~0.0038%). The authors suggested that the effectiveness of oxidation by the ozonation process is also affected by nonstarch content. However, in the present case, the sample high purity (~99%) ensure the starch reaction with ozone the only representative reaction.

The molecular size distribution through gel permeation chromatography is shown in Figure 2-C. The gel permeation chromatography is a size exclusion technique, whereby the molecules are separated according to their size. Starch molecules, in an eluant stream, are placed into a column filled with a gel with a specific porosity. During elution, the smaller molecules are retained in the pores, spending more time in the column and eluting later than the bigger ones (BRUMMER; CUI, 2005; HIZUKURI; ABE; HANASHIRO, 2006). Thereupon, highly branched and large-sized molecules, such as amylopectins, elute earlier than linear and small-sized molecules, such as amyloses.

In Figure 2-C, a decrease of the first peak and an increase in the second peak with increasing O₃ consumption are observed. Moreover, the peaks moved to right

when compared with native starch (0 mg $O_3 g_{starch}^{-1}$). These results indicate a reduction of large and branched molecules and an increase of smaller sized molecules due to ozonation. In the 270.7 mg $O_3 g_{starch}^{-1}$ of O_3 consumption, the first fraction peak is not evident and the second fraction is higher than the other samples, demonstrating that the large and branched molecules were reduced to small-sized molecules. Even so, both amylose and amylopectin were affected – in fact, this is also confirmed in Figure 2-D, whereby it is observed a reduction in the apparent amylose content as the O_3 consumption increases.

Therefore, the molecular size distribution demonstrates that the ozonation process not only oxidized the starch molecules but also had an important impact on molecular depolymerization. The degree of molecular cleavage is a function of the characteristics of the product (granule and molecular composition and properties) and process conditions (ozone concentration and gas flow, reactor design, dispersion concentration, temperature and O₃ consumption).

Castanha, Matta Junior, and Augusto (2017) ozonated potato starch in similar conditions used in this work, observing the same reduction of highly branched and large-sized molecules, as well as an increase in the amount of small-sized molecules. On the other hand, Matta Junior et al. (2019), performed mild ozonation of cassava starch, resulting in slight hydrolysis. As a consequence, they observed an increase of both first and second peaks, indicating the iodine affinity of the resulted molecules was increased.

Summarizing, the ozonation process results in a formation of carbonyl and carboxyl groups, as well as is responsible for the starch molecular depolymerization, once this starch modification process is responsible for the reduction of highly branched and large-sized molecules.

3.3. Starch granule characterization

3.3.1. Starch granule morphology and size distribution

The scanning electronic microscopy (SEM) and light microscopy (LM) of the native and ozonated starches are shown in Figure 3.



Figure 3. Morphology of native and ozonated cassava starch granules. The white and red line in the lower left corner of the images measures 100 µm.

Both native and ozonated cassava starch granules presented smooth surface and round shape with a truncated end on one side. The ozonated cassava starch granules did not present any irregular forms, roughness, pores, cracks or were broken. This indicates that the ozonation process did not cause relevant changes in the cassava starch granule surface and morphology.

Çatal and İbanoğlu (2012) modified wheat, rice, corn and potato starches with ozone, without noticing any changes for rice and wheat starches after the ozonation process. However, the authors noticed that the corn and potato starch granules' surfaces became rough and fibrous. In fact, Castanha, Matta Junior, and Augusto (2017) observed slight changes in the shape and surface of potato starch granules after the ozonation process. Klein et al. (2014) also did not notice any difference between the native and ozonated cassava starches morphology.

Even though the cassava starch shape and surface did not present any changes, it is important to highlight that the ozonation treatment was able to modify the starch molecular structure, as present in section 3.1.1.

The native and ozonated cassava starches particle size distribution is presented in Figure 4.



Figure 4. (A) Particle size distribution, (B) Volume-based and (C) area-based mean particle diameter of the native and ozonated cassava starches. Red bars indicate standard deviations. Variations followed by the same small letters do not differ significantly (p < 0.05).

In Figure 4-A, native starch has the highest frequency and 270.7 mg $O_3 g_{starch}^{-1}$ sample the lowest; however, both have a diameter of 25 µm, and as for 78.9 and 145.3

mg O₃ g_{starch}⁻¹, the granules' diameter was around 20 μ m. D [4,3] (Figure 4-B) did not present relevant changes between treatments. In D [3,2] (Figure 4-C), the 145.3 mg O₃ g_{starch}⁻¹ sample exhibited a slight reduction when compared with the native starch. Possibly this reduction is not necessarily related to the ozonation process, once the other ozonated samples did not present a significant change. This reduction is probably related to the irregular form and reduced size of cassava starch granules. These results indicate that the ozonation process had no important influence on the starch particle size distribution.

Castanha et al. (2019) noticed a decrease of the D[4,3] from the ozonated potato samples, but the D[3,2] values did not present a significant change. The authors stated that their results were due to the greater accessibility of the ozone to the larger granules during the processing. It is important to consider that the potato starch is one of the largest native granules, with dimensions ~60% bigger than cassava starch, which can explain why in the present work little variation on the granules' sizes was observed.

3.3.2. X-ray diffraction and relative crystallinity

The X-ray diffraction patterns of native and ozone-oxidized cassava starches, as well as their respective relative crystallinity (RC), are presented in Figure 5.



Figure 5. (A) X-ray diffraction pattern and (B) relative crystallinity (%) of native and ozone-oxidized cassava starches (calculated from 20 up to 30°). Red bars indicate standard deviations. Variations followed by the same small letters do not differ significantly (p < 0.05).

Generally, starch granules exhibit distinct types of diffraction patterns, such as A-type, B-type and C-type (a mixture of A and B-type), and these types indicate various

polymeric forms of the starch which differ in the packing of the amylopectin double helices (BULÉON et al., 1998; SINGH et al., 2003).

In X-ray diffraction analysis (Figure 5-A), the native and ozone-oxidized cassava starches presented peaks at 15°, 17°, 18° and 23°, being consistent with an "A" diffraction pattern. The same patterns were observed by other authors for cassava starch (KLEIN et al., 2014; MEI et al., 2015; HORNUNG et al., 2016; MATTA JUNIOR et al., 2019).

In this work, the RC (Figure 5-B) presented a slight decrease as the O_3 consumption increased. The RC of the 270.7 mg O_3 g_{starch}⁻¹ sample was ~11% lower than the native starch, for example. The changes in the RC can be attributed to the influence of the ozonation process on the amylopectin chains.

According to Miao, Zhang, and Jiang (2009), the RC differences between starches could be related to the orientation of the double helices within the crystalline area, the range of the interaction between the double helices, the crystal size and the number of crystalline zones, that are influenced by crystalline content and chain length. It is worth noting that the changes in RC are a function of both product characteristics and process conditions.

Castanha, Matta Junior, and Augusto (2017) observed no significant difference between the native and the ozonated potato starches RC, different from the results presented in this study. Klein et al. (2014), while studying the effects of ozonated cassava starches (for 60 min) at different pHs, also noticed a lower RC after ozonation, independently of the pH of the suspension. The authors stated the lower RC presented by the ozonated cassava starches demonstrates that ozone acts not only in the amorphous lamellae, but also in the semi-crystalline domain of starch. However, recent studies (LONDOÑO-RESTREPO et al., 2014, 2018; ALONSO-GOMEZ et al., 2016; RINCÓN-LONDOÑO; MILLAN-MALO; RODRÍGUEZ-GARCÍA, 2016) have questioned the reliability of RC evaluation, also using a new approach to evaluate the X-ray patterns. It demonstrates that the discussion about starch crystallinity is still open, and demands further understanding about the subject.

3.4. Starch properties and possible applications

3.4.1. Pasting Properties

The pasting properties of native and ozonated cassava starches are shown in Table 1 and Figure 6.

Table 1. RVA parameters for native and ozonated cassava starch. Peak Apparent Viscosity (PAV), Trough Apparent Viscosity (TAV), Relative Breakdown (RBD), Final Apparent Viscosity (FAV), Relative Setback (RSB) and Pasting Temperature (PT). Average \pm standard deviations; variations followed by the same small letters within a column do not differ significantly (p < 0.05).

| O ₃ Consumption _(mg O ₃ g _{starch} ⁻¹) | PAV (mPa.s) | TAV (mPa.s) | RBD (%) | FAV (mPa.s) | RSB (%) | PT (⁰C) |
|--|--------------|----------------|--------------|---------------|------------|------------|
| 0 | 4218 ± 88 ab | 1222 ± 13 a | 71.0 ± 0.3 d | 2052 ± 36.7 a | 67 ± 1.1 b | 65 ± 0.0 d |
| 78.9 | 4439 ± 121 a | 78 ± 3 b | 98.2 ± 0.1 c | 141 ± 6.5 b | 80 ± 3.1 a | 69 ± 0.9 c |
| 145.3 | 4097 ± 183 b | 44 ± 2 c | 98.9 ± 0.0 b | 67 ± 3.6 c | 54 ± 2.9 c | 70 ± 0.5 b |
| 270.7 | 3333 ± 188 c | 28 ± 2 d | 99.1 ± 0.0 a | 44 ± 2.6 d | 58 ± 5.6 c | 72 ± 0.5 a |



Figure 6. RVA curves of native and ozonated cassava starch.

According to Liu (2005), the peak apparent viscosity (PAV) takes place at the equilibrium point between swelling and granule rupture. As noticed in Table 1, the 78.9 and 145.3 mg $O_3 g_{starch}^{-1}$ samples did not present a significant PAV change, but the 270.7 mg $O_3 g_{starch}^{-1}$ sample exhibited a notable decrease. The PAV decrease indicates that the 270.7 mg $O_3 g_{starch}^{-1}$ sample presented a lower capacity to maintain their integrity.

Similar results were found in some studies (CATAL; BANOGLU, 2012, 2014; OLADEBEYE et al., 2013; CASTANHA; MATTA JUNIOR; AUGUSTO, 2017), which were associated with a granular "weakening" due to the partial cleavage of glycosidic bonds (decreasing the starch molecular chain-lengths). Moreover, according to Castanha; Matta Junior and Augusto (2017), it is also related to the replacement of the hydroxyl groups by carbonyl and carboxyl groups, decreasing the attractive hydrogen bonds between the molecules of modified starches, and substituting it to repulsive electrostatic interactions. However, Klein et al. (2014), studying the ozonation process in cassava starch at different pHs, observed higher PAV values at a pH of 6.5 and 9.5. The authors related their results to the cross-linking between some previously depolymerized starch molecules, increasing its molecular size. Oladebeye et al. (2018), investigating the effects of gaseous ozonation on pigeon pea, lima bean, and jack bean starches at different processing times (5, 10 and 15 min), notice an increase on the PAV values, and the authors associated these results to chemical crosslinking. However, the gel permeation chromatography (Figure 2-C) did not show evidence of crosslinking in the present work. On the other hand, some conditions of ozonation resulted in stronger gels (as described in the next section), which can be associated with interactions between hydroxyl-carbonyl and/or carbonyl-carbonyl groups. In fact, Zhang (2013) described the carbonyl groups are available to form strong hydrogen bonds with the hydroxyl groups on starch. This can explain the obtained results.

The relative breakdown (RBD) and relative setback (RSB) were calculated as proposed by Castanha, Matta Junior, and Augusto (2017).

The process of breakdown is defined by the granules rupture and subsequent polymer alignment. In Table 1, it is noted that, as the O_3 consumption increases, an increase in RBD values occurs, which indicates that the granules' rupture becomes more evident with O_3 consumption. These findings were in accordance with Castanha, Matta Junior, and Augusto (2017).

The setback represents the retrogradation tendency of starch molecules. The retrogradation process can be defined as a re-association between starch molecules (after the system being cooled), especially amylose (LIU, 2005). The rearrangement in starch molecules can be determined according to the affinity of hydroxyl groups of one molecule to another, especially the amylose, which has a huge affinity to form hydrogen bonds between hydroxyls and adjacent molecules (CASTANHA; MATTA

JUNIOR; AUGUSTO, 2017). However, starch retrogradation is not favored by chains that are too long or too short, which reduces the capacity of hydrogen bonds formation (WANG et al., 2015). In oxidized starches, the hydrogen bonds are affected in such a way that their tendency to retrogradation is reduced (RAPAILLE; VANHEMELRIJCK, 1997).

As observed in this study, the RSB of the 78.9 mg O₃ g_{starch}^{-1} sample was higher than the native cassava starch, which indicates that the 78.9 mg O₃ g_{starch}^{-1} molecules were more prone to re-associate than the native starch molecules. This is possibly due to the molecular depolymerization, forming smaller molecular chains, and its size favored the molecules' re-association. In relation to the 145.3 and 270.7 mg O₃ g_{starch}^{-1} ¹ samples, the RSB was lower than native and 78.9 mg O₃ g_{starch}^{-1} samples, indicating that the molecules of these starch samples were less prone to re-associate than the native and 78.9 mg O₃ g_{starch}^{-1} samples' molecules. These results can be attributed to higher molecular depolymerization (section 3.1.1, Figure 2-C and D) that possibly formed too small molecules, and to an increase of carboxyl and carbonyl groups (section 3.1.1, Figure 2-A). Therefore, the capacity of hydrogen bonds' formation was reduced, reducing the tendency to retrogradation. Matta Junior et al. (2019) also notice a reduction of RSB for ozonated cassava starch.

Castanha, Matta Junior, and Augusto (2017), on the other hand, presented an RSB increase for ozonated potato starches. The author suggested their molecular depolymerization led to a formation of small-sized molecules, presenting a more suitable size for re-association. We highlight again the balance among depolymerization (and the obtained relative size), carboxyl and carbonyl formation will occur differently according to both source and process characteristics, affecting differently the starch properties. In fact, it highlights again the need for further studies concerning different sources and process conditions. Chan, Bhat and Karim (2009), Oladebeye et al. (2013), Çatal and Ibanoğlu (2014), and Oladebeye et al. (2018) noticed a setback decreasing as the oxidation level increased. Klein et al. (2014), observed the lowest setback value in cassava starch treated at pH 3.5 and attributed the result to a disability of cleaved starch molecules to interact and retrograde. The samples treated at pH 6.5 and 9.5 showed a higher setback than the starch treated at pH 3.5, and the author related it to the cross-linking of the depolymerized molecules. Yet, all the mentioned authors did not consider using the RSB value, which made it difficult to compare the results.

The pasting temperature (PT) increased as the O₃ consumption increased. Castanha, Matta Junior, and Augusto (2017), also observed an increase in the PT, which they associated with the starch depolymerization since lower-size molecules require more energy to gelatinize when compared to larger-size molecules. However, Chan, Bhat and Karin et al. (2009) observed PT reduction for ozone-oxidized corn, sago and tapioca starches, which can be associated with mild process conditions, since an increase in PT occurred at the highest ozone generation time. Oladebeye et al. (2013), processing red and white cocoyam starches with ozone, and Çatal and Ibanoğlu (2014), processing wheat starch, also observed reductions in the PT. On the other hand, Klein et al. (2014) noticed that the PT of the ozonated cassava starches were similar to the native sample, which can be possibly related to the mild process conditions used by the authors.

Summarizing, the ozonation process affected the cassava starch pasting properties, which can be interesting for some industrial applications where the reduction of the viscosity is desired. In fact, according to lida et al. (2008), a starch gel with high viscosity is undesirable for spray-drying – once a high viscosity of the solution will encumber the spraying process. Another change observed in the ozonated cassava starches, with great industrial importance, is the reduction of the starch retrogradation – once a slow retrogradation can improve freeze-thaw stability, shelf life, cold-storage stability, and the product texture. Also, a slow retrogradation is efficient in retarding bread staling and improving bread crumb texture, improving the bread aspect and breadmaking functionality (BERTOLINI, 2009).

3.4.2. Hydrogel texture

Figure 7 illustrates two different aspects of the hydrogel firmness of native and ozonated cassava starch: force *versus* penetration depth of the starch gels before their disruption (Figure 7-A), and maximum force to initiate the starch gels' disruption (Figure 7-B). Moreover, Figures 8 and 9 exhibit the obtained hydrogels, representing their consistency.



Figure 7. Gel firmness of native and ozonated cassava starch prepared with water or citric acid solution (pH 3): (A) force versus penetration depth, (B) maximum peak force before disruption. Variations followed by the same small letters do not differ significantly (p < 0.05).



Figure 8. Consistency of starch hydrogels.



O₃ Consumption (mg O₃ g_{starch}⁻¹)

Figure 9. Native and ozonated cassava starch hydrogels after being removed from the mold.

Cassava starch is known by forming relatively weak gels. This can be visualized in Figure 9, once the gel cannot maintain its form after being removed from the mold.

Moreover, the native starch hydrogel was ~78% weaker when prepared in citric acid solution (pH 3) than when prepared with water (Figure 7). Also, acid gel presented a "liquid" form (Figures 8 and 9) when compared with the native starch hydrogel prepared with water (Figures 8 and 9). This result highlight cassava starch is greatly impaired by acid conditions normally observed in food products, limiting its application.

On the other hand, ozonated starches hydrogels were able to maintain their consistency in acidic conditions (Figure 8). This is a noticeable characteristic for industrial application, being here demonstrated for the first time, and demonstrating ozone processing can be an alternative to produce modified starches able to be used in acid foods.

Independently of the hydrogel pH (prepared with water or with citric acid), the 145.3 mg $O_3 g_{starch}^{-1}$ hydrogel required a higher force for disruption than the native, 78.9 and 270.7 mg $O_3 g_{starch}^{-1}$ samples (Figure 7-B). The 78.9 and 270.7 mg $O_3 g_{starch}^{-1}$ hydrogels disrupted before reaching a penetration depth of 10 mm (Figure 7-A). This indicates they are more plastics and stronger, which is confirmed in Figure 8. On the other hand, the 78.9 mg $O_3 g_{starch}^{-1}$ sample (prepared with water) presented a weaker

gel, even considering the native starch. The native and 78.9 mg O_3 g_{starch}-1 samples hydrogels did not disrupt in this assay, presenting a similar tendency.

It is worth mentioning the 145.3 and 270.7 mg $O_3 g_{starch}^{-1}$ gels maintained their shapes after removed from the mold (Figure 9), and it was possible to obtain hydrogels ~280% stronger than those from native cassava starch (after O_3 consumption of 145.3 mg $O_3 g_{starch}^{-1}$).

Stronger gels are highly desired for industrial application, in special considering cassava starch. Therefore, the obtained results are very positive, expanding the possible uses of this source.

Chan et al. (2011) also observed stronger gels for corn and sago ozonation starches. However, the authors could not measure the ozonated cassava starch gels, because they exhibited weak formation, which made it difficult to determine their firmness – and thus highlighting the obtained results in the present work. Castanha, Matta junior, and Augusto (2017) also notice stronger gels for ozonated potato starch. On the other hand, Oladebeye et al. (2013) and Oladebeye et al. (2018) observed a reduction of the firmness of the gel for ozone processed starches samples.

In native starches, the ratio of amylose/amylopectin influences the final gel strength, since amylose it is known for the high tendency to produce hard gels and strong films, and amylopectin, after gelatinization, is produces flexible gels and weak films (PÉREZ; BERTOFT, 2010). However, when modification treatment is applied in starches, the ratio amylose/amylopectin may not be the only factor that will influence the final gel strength, since it occurs molecular depolymerization, with the formation of intermediate molecules. Moreover, the formed carbonyl and carboxyl groups also influence the interactions among the molecules. In fact, the final gel strength is a consequence of the molecular network after retrogradation, whose association, alignment and interactions will be a balance between the changes on molecular size and chemical characteristics (through the carbonyl and carboxyl groups) – being, therefore, a function of both product characteristics and process conditions.

Castanha, Matta junior, and Augusto (2017) related the increase of gel firmness with molecular depolymerization and increased mobility of molecules, whose size presents an appropriate distribution for re-association. At stronger conditions of ozonation, however, the intense molecular depolymerization compromised the re-association tendency, as well as carbonyl and carboxyl groups resulted in molecular repulsion – and, thus, reducing the gel strength. It explains the reason why some

processing conditions increased the starch gel firmness (such as O_3 consumption of 145.3 mg O_3 g_{starch}⁻¹ in the present work), while higher O_3 consumption reduced it (such as O_3 consumption 270.7 mg O_3 g_{starch}⁻¹).

Therefore, starch ozonation can be useful in applications where the gel consistency is important, such as in the production of desserts (such as flans and puddings), and meat products (such as sausages). Moreover, modified starches can be applied in acid food, such as tomato paste, sauces, and jelly candies.

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

The parameter water absorption index (WAI) represents the weight of gel formed per gram of dry starch and water solubility index (WSI) is the percentage of the matter in the supernatant from the WAI analysis (XIE; LIU; CUI, 2005).

The WSI and WAI results of the cassava starches samples are shown in Figure



Figure 10. (A) Water solubility index and (B) water absorption index of the native and ozonated cassava starches at different temperatures. The dots are the mean values, the vertical bars are the standard deviation. The curves are traced to facilitate interpretation.

The starch WSI increased by increasing both ozonation time and temperature (Figure 10-A). The solubility of the ozonated samples presented a more significant increase at temperatures above 70 °C.

During the gelatinization process, when heated in the presence of water, the starch granules absorb water and swell, and some starch components (mostly amylose) leach out and solubilize (MOORTHY; SAJEEV; ANISH, 2018). Therefore, the increase of solubility observed for higher temperatures and O_3 consumption is

possibly related to the small chains of amylose and amylopectin arising from the depolymerization during the ozonation process, which are easily leached. This WSI increase can be considered an advantage for industrial application, since, according to Castanha et al. (2019), products like instant soups and chocolate powder demand easy solubilization.

Chan, Bhat, and Karim (2009), and Oladebeye et al. (2013) also observed an increase of WSI for ozonated samples and they related their results to depolymerization and structural weakening of the starch granules, after the ozonation process. On the other hand, Castanha et al. (2019) associated the solubility increase to a molecular cleavage and an increase in electronegative groups, and these were responsible for disrupting starch granules.

The starch water absorption, on the other hand, depends on the presence of hydrophilic groups which bind water molecules and on the gel-forming capacity of macromolecules (GOMEZ; AGUILERA, 1983). In native starch, it is also related to amylopectin to amylose ratio, where amylopectin contributes to high water absorption, while high amylose content decreases it (MANI; BHATTACHARYA, 1998; KOTOKI; DEKA, 2010). The water absorption index (WAI) (Figure 10-B) decreased as the temperature increased for the ozonated starches. The native and ozonated samples showed a similar water absorption at 60 °C. Considering the O₃ consumption, it was noticed a decrease of the WAI of the ozonated samples, when compared to the native cassava starch. Possibly this reduction of WAI is related to a weaker molecular network of the ozonated starch better water retention.

Some authors (Castanha et al. 2019; Oladebeye et al. 2013; Chan, Bhat, and Karim 2009) noticed a WAI decreasing with increasing ozonation time. Chan, Bhat and Karim (2009), and Oladebeye et al. (2013) associated their results to structural disintegration within the starch granule during the oxidation process.

3.4.4. Paste Clarity

The results of paste clarity of the cassava starch are present in Figure 11.



Figure 11. Paste clarity of the native and ozonated cassava starches during 20 days of storage at 5 °C. The dots are the mean values, the vertical bars are the standard deviation. The curves are traced to facilitate interpretation.

The ozonated cassava starches presented a paste much clearer than the native starch, which is a result of high industrial interest. Moreover, all ozonated cassava starches presented a slight decrease after one day of storage, but after twenty days of storage, the processed samples presented a tendency to maintain their clarity. Even so, the paste clarity over the storage time can be considered high stable and much higher on ozonated starches than in the native one.

Starch paste clarity varies significantly according to the source of the starch, being altered by modification processes (CHEN; KAUR; SINGH, 2018). Some factors that influence the clarity of starch pastes are: Amylose content (responsible for paste opacity), presence of phosphate-monoester (increase paste clarity), presence of reducing sugar (increase paste clarity) and electronegative repulsion (increase paste clarity) (CRAIG et al., 1989; VISSER et al., 1997; SITOHY; RAMADAN, 2001). The presence of carboxylic groups in starch molecules (section 3.1), which are electronegative charged and bulkier than hydroxyl groups, causes repulsion between the molecules, which are kept at a specific distance. Consequently, paste clarity increases. In fact, according to Xie, Liu and Cui (2005), the carboxyl and carbonyl groups interfere with the tendency of amylose to retrograde and associate, producing starch pastes with higher stability and clarity. However, this repulsion between the molecules – that promotes a clearer starch paste – could result in a weaker starch

hydrogel as shown in section 3.3.2. Perhaps, these results could be associated with interactions of hydroxyl-carbonyl and/or carbonyl-carbonyl, resulting in stronger hydrogels.

Castanha, Matta Junior, and Augusto (2017) presented similar results for ozonated potato starch, although the authors did not evaluate paste clarity behavior during a storage period.

In relation to the industry perspective, starch with a high paste clarity can be applied in textile and paper industries. It is also suitable for the food industry, since a clearer gel paste is excellent for fruit pie fillings, as also for canned products, whereby clarity is essential to preserve the attractive appearance of vegetables.

4. CONCLUSIONS

The ozonation process was able to form carbonyl and carboxyl groups, as well as to cause molecular depolymerization, which generated changes in the cassava starch properties. For instance, pasting properties, gel texture, water absorption index (WAI), water solubility index (WSI) and paste clarity were affected. The changes in starch properties were correlated with those on the structure. It is important to highlight some of these changes since they can benefit the cassava starch used in the industry, such as follows: reduction of the viscosity, reduction of the starch retrogradation, stronger hydrogels – in special at acid conditions -, increase of WSI and reduction of WAI, and higher paste clarity. In this way, the ozonation process can expand the possibilities for cassava starch industrial applications.

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Chapter 3: Ozone modification of arracacha starch: Effect on structure and functional properties

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ABSTRACT

Arracacha starch presents interesting properties, such as a relatively low temperature of gelatinization and high paste clarity when compared with other commercial starches. Those characteristics can be very useful for industrial applications. However, the native arracacha starch presents some drawbacks that limit its further application, such as a weak gel and a high consistent paste. Therefore, modification techniques can expand its utilization. Ozone technology is a promising modification method, once studies reported that ozonation can decrease the apparent viscosity and increase the gel strength of different starch sources. Therefore, this work aimed to enhance arracacha starch properties (for instance, gel strength) by using ozone technology, in order to provide an alternative to the traditional oxidation chemical processes and to improve this starch's industrial applications. To achieve this, the effects of ozonation were evaluated in starch structure (granule and molecules) and properties, and possible industrial applications were suggested. The ozonation process promoted structural changes - reduction in the size of the molecules, formation of carbonyl and carboxyl groups, and an increase of reducing sugar content - that significantly affected the arracacha starch properties. Among the main results, it is worth highlighting the improved stability of paste clarity and hydrogels up to 250% stronger than the native one. Therefore, the ozonation process demonstrated to expand the arracacha starch industrial use.

Keywords: Arracacha starch; modification; ozone technology; green technologies.

1. INTRODUCTION

Arracacha (*Arracacia xanthorrhiza* Bancroft) is a root originally from the Andean South America region, and its production is mainly concentrated Colombia, Venezuela, Ecuador, Peru, Brazil and Bolivia (ALBANO; FRANCO; TELIS, 2014; LONDOÑO-RESTREPO et al., 2018; SANTOS et al., 2018).

In general, the arracacha root is an "underutilized" crop (BLAS; HERMANN; BAUDOIN, 2008; LEIDI et al., 2018), presenting a low consumption and commercialization, even considering the production of bread, chips, soups, and for starch extraction (ROCHA et al., 2011; LONDOÑO-RESTREPO et al., 2018). In fact, the isolation of starch from arracacha roots is an advantageous option to increase its commercialization, since the roots present a high content of starch, which is a great potential for industrial use (LEONEL et al., 2016; CASTANHA et al., 2018; LONDOÑO-RESTREPO et al., 2018). In fact, Arracacha starch presents some interesting properties for specific industrial application, that will be mentioned below.

Arracacha starch has already been extracted and characterized by different researchers (ALBANO; FRANCO; TELIS, 2014; LEONEL et al., 2016; CASTANHA et al., 2018; LONDOÑO-RESTREPO et al., 2018). Castanha et al. (2018), for instance, evaluated starches from two varieties of Arracacha roots of the Andean Peruvian region and described some interesting characteristics for those starch samples, such as a high-water absorption and holding capacity, a low gelatinization temperature, and a high paste clarity compared with other commercial starches, i.e., cassava starch (NWOKOCHA et al., 2009). Those characteristics can be very useful for several industrial applications, being especially attractive for the food industry, since those properties are presented in a natural source – for instance, a starch with high paste clarity is excellent for using it in fruit pie fillings (RAPAILLE; VANHEMELRIJCK, 1997).

However, the native arracacha starch presents some drawbacks that limit its application in the industry, such as a weak gel and a paste with a high apparent viscosity (CASTANHA et al., 2018; LONDOÑO-RESTREPO et al., 2018), which can impair its applications. Thus, despite the interesting properties presented by the native arracacha starch, its modification can be interesting in order to enhance its further industrial uses.

In fact, there are a few works in the literature that applies methods to modify arracacha starch (MATSUGUMA et al., 2009; MEDINA; PARDO; ORTIZ, 2012; MORAES; BRANZANI; FRANCO, 2014). However, the modification technologies used in these works present some disadvantages that can impair their industrial use. For example, Moraes, Branzani, & Franco (2014) modified arracacha (and cassava) starch using the heat-moisture treatment, at 90 °C during 8 h, which is a long processing time, being therefore costly for the industry. Matsuguma et al. (2009) and Medina, Pardo, & Ortiz (2012), used chemical modification agents that generate toxic wastes in the environment, being necessary treatments to reduce the toxicity, which limits the use "clean labels" by the industry.

Therefore, it would be interesting to use a "clean label" modification process to overcome the limitations of native arracacha starch, expanding its application. In this

way, we selected the ozone technology - an oxidation method of starch modification - to focus on this work.

Oxidation is a chemical method of starch modification that is carried out by a reaction with an oxidant agent under controlled conditions (WANG; WANG, 2003). This is a common method used to modify native starches, where the hydroxyl groups present in the starch molecules are oxidized to carbonyl and carboxyl groups (PANDISELVAM et al., 2019). The main chemical oxidizing agents used for starch oxidation are hydrogen peroxide, sodium hypochlorite, and sodium periodate (PANDISELVAM et al., 2019). However, these oxidizing agents produce toxic by-products.

On the other hand, the ozone technology is an attractive alternative to the traditional oxidation chemical processes, as it is considered a "environmentally friendly" and "green" technology (ozone decomposes in oxygen). In addition, ozone has been studied in different starch sources at different conditions (OLADEBEYE et al., 2013; CASTANHA; MATTA JUNIOR; AUGUSTO, 2017; PANDISELVAM et al., 2017; OLADEBEYE et al., 2018; CASTANHA et al., 2019; MATTA JUNIOR et al., 2019; PANDISELVAM et al., 2019), and is mostly reported to cause a decrease in the apparent viscosity and an increase of the gel strength of different starch sources. As those are the main drawbacks related to the arracacha starch samples, the ozone would be a suitable technology to that end.

Therefore, this work aimed to enhance arracacha starch properties (for instance, gel texture) by using ozone technology, in order to provide an alternative to the traditional oxidation chemical processes and to improve these starch's industrial applications. In this way, the effects of ozonation were evaluated in starch morphology, structure and properties. Then, possible industrial applications for the modified arracacha starch were suggested.

2. MATERIAL AND METHODS

2.1. Starch sample and ozonation process

We worked with the same starch sample isolated from a yellow variety of Peruvian arracacha roots and characterized in a previous work of our research group research group (CASTANHA et al., 2018).

The ozonation process was carried out in laboratorial scale, using the same system described by Castanha, Matta Junior, & Augusto (2017). In short, ozone was

produced in an ozone generator unit (Ozone & Life, O&G Model RM 3.0, São José dos Campos, Brazil) by the coronal-discharge method from industrial oxygen (95% purity). The native starch was added in 400 mL of distilled water (10% m/m on dry basis), placed in a glass reactor (2 L capacity, 56 cm of height, 6 cm of internal diameter) and processed for 15 and 30 minutes, being constantly stirred with a magnetic stirrer. The gas flow in the reactor was maintained constant at 1 L·min⁻¹, and the ozone concentration was measured by an ozone monitor (2B Technologies, Model 106-H, USA), and its concentration was kept at 47 mg O₃·L⁻¹.

After processing, the suspensions were maintained at rest for one hour and the supernatant was discarded. The starch was dried in an air circulation oven at 35 °C until ~12% of moisture content (on wet basis). The dried samples were then milled in a mortar, sieved in a 60-mesh sieve (250 μ m) and kept stored in glass flasks for further analysis. An unprocessed starch sample was used as control (0 min).

2.2. Starch granular characterization

2.2.1. Granule morphology

The arracacha starch granules morphology was evaluated by scanning electronic (SEM) and light microscopies (LM).

The SEM was carried out using a FEI Inspect F50, (Japan) operating at an acceleration voltage of 2.0 kV and available at LNNano, Campinas, Brazil. The samples were prepared as follows: a double sticky carbon tape was fixed on a circular aluminum stub, and dry starch was sprinkled on the tape. A brush was used to ensure a uniform layer of powder particles. The stubs were coated with gold (~4 mm) and then evaluated in the microscope using an Everhart-Thornley Detector (ETD) for secondary electrons. The scale of the SEM images was directly provided by the equipment.

A light microscope (model L1000, Bioval, Curitiba, Brazil), with a 20 W halogen lamp, was used to observe the starch granules' shape, surface and edges. The samples (0.1 g) were dispersed in 10 mL of distilled water with 50 µL of Lugol. Then a drop of this solution was placed on a glass slide covered by a glass coverslip. The magnification used to obtain the images was of 400x. To observe the Maltese crosses, a polarized light filter was coupled to the system. The scale of the LM images was obtained by using a glass slide that had a standard scale on it. This glass slide was placed in the microscope, and the same magnification used to examine the starch granules was applied to observe the standard scale. Then a picture was taken of this standard scale. After taking the picture, it was used the software Adobe Illustrator (version 24.1.1, Adobe Systems, USA) to trace a line in the scale. Then, this line was placed in the LM images.

2.2.2. Particle size distribution

The particle size distribution was evaluated using a Laser Analyser (Partica LA-950V2 Laser Particle Size Analyser HORIBA, Japan) and to analyze the data, it was used the LA-950 software for Windows (HORIBA, Japan). The arracacha starch granules were dispersed in ethanol (99.5%). Before the analysis, the samples were placed in an ultrasound bath for 3 minutes. The volume-based mean diameter (D [4,3]) and the area-based mean diameter (D [3,2]) were determined according to Equations 1 and 2 (RAWLE, 2003), respectively - Where n_i is the number of particles of diameter d_i .

$$D[4,3] = \frac{\sum_{i} n_{i} d_{i}^{4}}{\sum_{i} n_{i} d_{i}^{3}}$$
(1)

$$D[3,2] = \frac{\sum_{i} n_{i} d_{i}^{3}}{\sum_{i} n_{i} d_{i}^{2}}$$
(2)

2.3. Starch molecular characterization

2.3.1. pH

The pH was evaluated using a calibrated potentiometer (Tecnal, TEC-5 mode, Piracicaba, Brazil). The starch samples (1 g, on dry basis) were suspended in 10 mL distilled water, being constantly stirred with a magnetic stirrer (ADOLFO LUTZ INSTITUTE, 2008).

2.3.2. Carbonyl and carboxyl groups content

The carboxyl group content was carried out as described by Chattopadhyay, Singhal, & Kulkarni (1997), with modifications. The starch (1 g, on dry basis) was dispersed in 25 mL of 0.05 M HCl and stirred for 30 min using a magnetic stirrer. Then the samples were vacuum filtered using a qualitative filter paper ($205 \mu m$) and washed with 400 mL of distilled water, in order to remove the residual acid. The obtained starch was suspended in 300 mL of distilled water, then brought to a boiling water bath with continuous manual stirring for 15 minutes to ensure gelatinization. The hot gelatinized

starch was then titrated with 0.002 M NaOH using an automatic titrator (Plus Titrino 848, Metrohm, Herisau, Switzerland) until pH 8.3. An unmodified starch was used as a blank. The result was expressed in the quantity of carboxyl groups per 100 glucose units (COOH/100 GU). Carboxyl group content was calculated according to Equation 3.

$$\frac{\text{COOH}}{100\text{GU}} = \frac{[(\text{Vs}-\text{Vb})\cdot\text{M}\cdot0.045\cdot100]}{\text{W}}$$
(3)

Where:

Vs = the volume of NaOH required for the ozonated samples;

Vb = the volume of NaOH used in the blank;

M = molarity of NaOH;

W = the sample mass on dry basis.

The carbonyl group content analysis was performed according to the methodology described by Smith (1967). 2 g (on dry basis) of starch samples were suspended in 100 mL of distilled water. The suspension was placed in a boiling water bath and manually stirred for 20 minutes (until complete gelatinization), and the obtained gel was cooled to 40 °C, adjusted to pH 3.2 with HCl 0.1 M. An aliquot of 15 mL of hydroxylamine solution (5% of hydroxylamine hydrochloride and 20% of 0.5 M NaOH solution in distilled water) were added to the sample. Then the samples were placed in a 40 °C water bath with stirring for 4 h. Thereafter, the excess of hydroxylamine was titrated with 0.1 HCl, using an automatic titrator (Metrohm, 848 Tritino plus, Herisau, Switzerland), until pH 3.2. A blank, without sample, was prepared with all reagents. The result was expressed in the quantity of carbonyl groups per 100 glucose units (CO/100 GU). Carbonyl group content was calculated according to Equation 4.

$$\frac{CO}{100GU} = \frac{[(Vb - Vs) \cdot M \cdot 0.028 \cdot 100]}{W}$$
(4)

Where: Vb = the volume of HCl used in the blank; Vs = the volume of HCl required for the sample;

M = the molarity of HCI;

W = the sample mass on dry basis.

2.3.3. Gel permeation chromatography

The molecular size distribution profile of the starch molecules was evaluated by gel permeation chromatography (GPC), according to the methodology of Song & Jane (2000), with modifications. A GE XK 26/70 column (2.6 cm diameter and 70 cm high) packed with Sepharose CL-2B gel (Sigma, Sweden) was used. Starch samples (0.1 g, on dry basis) were dispersed in 10 mL of 90% dimethylsulfoxide (DMSO, Labsynth, Brazil), heated in a boiling water bath for 1 h and kept at 25 °C for 16 h under constant stirring. An aliquot of 3 mL was then mixed with 10 mL of absolute ethanol, to precipitate the starch molecules. Thereafter, the solution was centrifuged for 30 min at 3000 g. The supernatant was discarded, and the precipitated starch was dissolved in 9 mL of boiling distilled water, then 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 fractions of 4 mL of the eluted solution in different tubes. The samples were evaluated by the blue value method, as described by Juliano (JULIANO, 1971): to prepare an I₂/KI solution, 0.1 g of I₂ and 1.0 g of KI where dissolved in 100 mL of acetate buffer 0.2 M at pH 5.0. Then, 100 µL of I₂/KI solution were added in the fractions, then the samples were evaluated using a spectrophotometer at a wavelength of 620 nm (spectrometer Femto, Model 600S, São Paulo, Brazil).

2.3.4. Apparent amylose content

The apparent amylose content was performed according to the ISO methodology (1987). A standard curve was made by using standard amylopectin (A8515) and amylose (A0512 type III), both from Sigma-Aldrich Pty Ltd. (Casle Hill, Australia). Starch samples (0.1 g, on dry basis) were dispersed in 1 mL of ethanol 95% and 9 mL of NaOH 1 mol·L⁻¹. Then, the samples were gelatinized in a boiling water bath for 10 min and cooled at room temperature. An aliquot of 18 mL of gelatinized sample was mixed with 2 mL of NaOH 0.09 mol·L⁻¹. Thereafter, an aliquot of 5 mL of the solution was placed in a volumetric flask of 100 mL containing 1 mL of acetic acid

1 mol·L⁻¹. The volumetric flask was stirred, then it was added 2 mL of iodine solution (0.2% I₂, 2% KI, in distilled water) and the flask was filled with distilled water. After 20 minutes, the samples were evaluated in a spectrophotometer at a wavelength of 620 nm (spectrometer Femto, Model 600S, São Paulo, Brazil).

2.3.5. Reducing sugar content

The reducing sugar content was evaluated according to Somogyi-Nelson colorimetric method (NELSON, 1944; SOMOGYI, 1945). The starch (1 g, on dry basis) was suspended in 50 mL of distilled water, then it was added 5 mL of concentrated sulfuric acid. Afterward, the samples were heated in a water bath at 50 °C for 10 min, being manually stirred for 10 min. The samples were cooled and their pH was adjusted to 8.0 using NaOH 5 N. Then, distilled water was added until reaching a volume of 200 mL, being then filtered using a qualitative filter paper (205 µm). As described by Somogyi (1945), an aliguot of 1 mL of the filtered solution was transferred to a 10 mL tube and 1 mL of alkaline copper reagent was added, being the tube stirred and subsequently heated for 10 min in a boiling water bath. The tubes were then cooled in cold water and 1 mL of the arsenomolybdate reagent (prepared as detailed by Nelson (1944)) was added. The solution was then diluted in distilled water until reaching 10 mL, vigorously stirred and finally quantified in a spectrometer at 535 nm wavelength (spectrometer Femto, Model 600S, São Paulo, Brazil). The blank (without starch) was prepared using only distilled water and the Somogyi-Nelson reagents. A standard curve was previously prepared using solutions at different glucose concentrations (ranging from 0 to 100 µg of glucose per 1 mL).

2.4. Starch properties

2.4.1. Pasting properties

The pasting properties analysis was carried out using RVA-4 (Newport Scientific Pvt. Ltd., Australia, with the Thermocline for Windows software, version 3.0) using 3 g starch (corrected to 14% moisture in wet basis) in 25 g of distilled water. Then, they were analyzed according to a programmed heating, retention and cooling cycle under constant shear: Firstly, the suspension was held at 50 °C for 1 min, then heated to 95 °C, being then kept at 95 °C for 5 min, followed by cooling to 50 °C, and finally hold at

50 °C for 2 min. The relative breakdown (RBD) and relative setback (RSB) parameters were calculated as proposed by Castanha, Matta Junior, & Augusto (2017).

2.4.2. Gel strength

The starch hydrogels were obtained after the RVA analysis. The gels were stored at 5 $^{\circ}$ C for 24 h in a plastic recipient (40 x 20 mm, diameter x height) placed in a desiccator with water at the bottom to ensure uniform moisture. The gel strength of the starch gels, was evaluated by a puncture assay using a Texture Analyzer (TA.XT Plus, Stable Micro Systems Ltd., Surrey, UK) with a load cell of 50 kgf (490.3 N). A cylindrical probe (P/0.5R, 12.7 mm of diameter) was used to puncture the samples until the distance of 10 mm at 1 mm·s⁻¹.

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

The WAI and WSI were evaluated as described by Anderson, Conway, & Peplinski (1970), with some modifications. Starch samples (0.5 g, on dry basis) were dispersed in 6 mL of distilled water in pre-weighed centrifuge tubes. The tubes were placed in a shaking thermal bath for 30 min at different temperatures (45, 50, 55, and 60 °C), and then centrifuged at 3000 *g* for 10 min. The supernatant was placed in a pre-weighed glass Petri dish, dried at 105 °C for 16 h, then cooled and weighed. The precipitated retained in the tube was also weighed. The WAI (Eq. (5)) and WSI (Eq. (6)) were calculated, as follow:

WAI
$$\left(\frac{g \text{ water}}{g \text{ starch}}\right) = \frac{[PR - (MS - DS)]}{(MS - DS)}$$
 (5)

WSI (%) =
$$\left(\frac{\mathrm{DS}}{\mathrm{MS}}\right)$$
. 100 (6)

Where,

PR = mass of the precipitated retained;MS = mass of the sample, on dry basis;DS = mass of the dried supernatant.

2.4.4. Paste Clarity

Paste clarity was evaluated according to the method proposed by Aplevicz & Demiate (2007). The starch samples were mixed with 50 mL of distilled water and 0.5 g of starch in test tubes with screw cap. Then, the tubes were kept in a boiling water bath for 30 min and stirred individually 5 times every 5 minutes. After the thermal bath, the samples were stored at 5 °C for 20 days, to evaluate the paste clarity stability during this period. The paste clarity measurements were carried out at the following days: 0 (the day the paste was made), 1, 3, 5, 8, 12, 16, and 20th. The paste clarity was evaluated by transmittance measurement (T%) in a spectrometer at 650 nm wavelength (spectrometer Femto, Model 600S, São Paulo, Brazil).

2.5. Experimental design and statistical analysis

The experimental design was completely randomized. Each processing time was made in triplicate and the samples were analyzed in duplicate. Data were assessed by the analysis of variance (ANOVA) and comparisons of averages for treatments were made using Tukey's test using the software Minitab version 18 (Minitab, LLC., USA).

3. RESULTS AND DISCUSSION

3.1. Starch granular characterization: morphology and particle size distribution

Figure 1 presents the scanning electronic microscopy (SEM) and light microscopy (LM) containing the Maltese cross of the native and ozonated arracacha starches.



Figure 1. Morphology of native and ozonated arracacha starch granules, evaluated using both electronic and light microscopy. The white lines measure 5 µm and red lines measure 100 µm.

The native arracacha starch granules present a round or polygonal-shape, with some fissures and cracks on their surfaces, as illustrated by the SEM images. This unevenness in the starch granules' surface is possibly related to the fact that their granules are closely packed in the cells of the arracacha roots in a "Lego-like" conformation (LONDOÑO-RESTREPO et al., 2018). In general, as observed in Figure 1, the ozonation process did not change the arracacha starch granules' surface. Also, no changes were observed in the Maltese crosses of the ozonated arracacha starches, indicating that the ozonation process was not able to cause important changes in the granule morphology.

Klein et al. (2014) reported similar results regarding the surface of cassava starch submitted to ozone treatment (60 minutes of processing time, at different pHs), that is, no changes were observed after processing. However, some authors reported changes on the starch's surface after ozonation, which can be related to both starch source and process conditions. Castanha, Matta Junior, & Augusto (2017) noticed minor changes in the potato starch granules' morphology, which presented fissures and pores after their ozonation (processing times were 15, 30, 45 and 60 minutes). Çatal & İbanoğlu (2012) also observed changes in ozonated (processing time was 60

minutes) corn and potato starches, although no changes were observed in the processed rice and wheat starches. The authors also stated that the Maltese cross of all ozonated starches sources did not exhibit any changes, and this is in accordance with the present work. However, it is important to emphasize that the starch source and the processing conditions play an important role in the observed results. For example, smaller and more heterogeneous granules (as cassava, arracacha and maize) are more difficult to evidence possible surface changes than bigger and smooth surfaces granules (such as potato).

The particle size distribution of native and ozonated arracacha starches is shown in Figure 2. The processed samples presented a slightly broader variation of granules' sizes, which was evidenced by both size distribution frequency (Figure 2-A) and by the D[4,3] (volume-based mean) and D[3,2] (area-based mean) values (Figures 2-B and 2-C).



Figure 2. (A) Particle size distribution, (B) volume-based mean particle diameter and (C) area-based mean particle diameter of the native and ozonated arracacha starches. Red bars indicate standard deviations. Variations followed by the same small letters do not differ significantly (p< 0.05).

The D [4,3] is influenced by large particles, while the D [3,2] is influenced by smaller ones (LOPEZ-SANCHEZ et al., 2011). Therefore, the results from Figure 2 suggest a small increase in the size of bigger particles. This small increase could be associated with partial aggregation of granules, as described by Castanha et al. (2019). However, the present work has employed a dynamic system to evaluate the PSD, with constant stirring. Therefore, although possible, we judge aggregation as unprovable. Another possibility to explain this small increase can is a partial swelling of the starch granules due to the ozonation process (in water), which possibly can change the amylose and amylopectin packed arrangement. However, we highlight the differences in granule size were almost negligible.

Therefore, our results indicate that the 15 and 30 min ozone treatments promote only small changes in arracacha starch granule morphology, which highlights the importance to evaluate if this technology is able to modify the samples' molecular structure, as shown in section 3.2.

3.2. Starch molecular characterization: Carbonyl and carboxyl contents, pH, molecular size distribution, apparent amylose and reducing sugar contents

Both native and ozonated arracacha starches' structure was evaluated through different approaches, which are presented in Figure 3: carbonyl and carboxyl groups content (Figure 3-A), pH (Figure 3-B), molecular size distribution (Figure 3-C) and apparent amylose and reducing sugar contents (Figure 3-D).



Figure 3. Carbonyl and carboxyl groups content (A), pH (B), molecular size distribution profile based on the blue value method (C) and apparent amylose and reducing sugar contents (D) of the native and ozonated arracacha starch samples. The red * symbol indicates that the carboxyl content is considerate zero, since native starches do not have this compound. (D) The circles and squares represent the mean values. Red vertical bars are the standard deviations. Variations followed by the same letters do not differ significantly (p < 0.05).

In Figure 3-A, the 15 min ozonated starch did not exhibit a significant change for carbonyl group content, but after 30 min of processing, it was noticed an increase.

The carboxyl group content increased in both process conditions. The presence of the carboxyl group increases the acidity of the starch samples, which resulted in the reduction of the pH of their suspensions (Figure 3-B).

Other works also found similar results (CHAN: BHAT: KARIM, 2009; CHAN et al., 2012; OLADEBEYE et al., 2013; CASTANHA; MATTA JUNIOR; AUGUSTO, 2017; OLADEBEYE et al., 2018). In fact, the presence of these groups is expected after oxidative processes, such as the ozonation process, since the oxidation of the hydroxyl groups (probably at the carbons 2, 3 and 6) forms carboxyl and carbonyl groups (WURZBURG, 1986). However, some authors noticed higher values of carboxyl groups in ozonated samples. For example, Klein et al. (2014), studying the effects of ozonation technology at different pH in cassava starch, observed that carbonyl group content was lower than the carboxyl group content. In their research, the maximum values of carbonyl and carboxyl group content were found at pH of 9.5 (0.011 and 0.028%, respectively), and are lower than in this study. Moreover, Matta Junior et al. (2019), investigating the potential use of the ozonation process to improve the ovenexpansion of cassava starch. They noticed similar content of carboxyl and carbonyl groups (~0.08 and 0.09%, respectively), possibly their findings are related to the mild processing conditions. These results indicate different oxidation levels. When we compare our results with other researches that used other oxidative agents of modification (sodium hypochlorite and hydrogen peroxide) (ZHU et al., 1998; SÁNCHEZ-RIVERA et al., 2005; SANGSEETHONG; LERTPHANICH; SRIROTH, 2009; SANGSEETHONG; TERMVEJSAYANON; SRIROTH, 2010; ZHOU et al., 2016), the degree of modification was low (carbonyl and carboxyl groups' content was low). Therefore, the ozonation process of this work can be considered a mild oxidative process. However, ozonation greatly affected the molecular size distribution, as presented in this section (Figure 3-C). Even though the degree of modification was low, the ozonation technology was able to modify the arracacha starch and enhance its properties (section 3.3.2).

The molecular size distribution profile is presented in Figure 3-C. Gel permeation chromatography (GPC) is a size-exclusion technique, where the polysaccharide molecules are separated mainly by their sizes (HIZUKURI; ABE; HANASHIRO, 2006). Starch molecules elute through a column filled with a gel with a specific porosity: bigger molecules elute first, normally associated with amylopectin, while the smaller ones (such as amylose) are retained in the pores and remain in the

column for a longer period (BRUMMER; CUI, 2005; HIZUKURI; ABE; HANASHIRO, 2006). Therefore, in a GPC profile (such as Figure 3-C), the first fraction peak represents the bigger molecules of the starch, whereas the second fraction peak represents the smaller molecules. However, in starch granules, we can find amounts of amylose slightly branched and with a large chain, which elutes in the first fraction, as also amylopectin with small size, which elutes in the later fraction (CASTANHA et al., 2018).

The molecular size distribution profile of the arracacha starch samples (Figure 3-C) presented a decrease of the first peak fraction, with a gradual increase of the second fraction, with increasing ozonation time. Also, the second peak of ozonated samples slightly moved to the right. These results are related to the reduction of bigger molecules (decrease of the first fraction), with a consequent increase of smaller molecules (increase of second fraction) – that indicates a molecular depolymerization of the ozonated starches. This depolymerization can be confirmed in Figure 3-D, whereby a reduction of apparent amylose content is noticed with the increase of ozonation processing time.

In a first seem, the reduction in the apparent amylose values can be interpreted as contradictory with the results of molecular size distribution, which demonstrates an increase of smaller molecules. However, it is important to highlight the differences between both analyses. In the apparent amylose analysis, all the molecules that react with iodine are colored – highlighting the term "apparent". In native starches, mainly amylose is colored with iodine, justifying this analysis. However, iodine can form complex with the branched-chain of amylopectins, overestimating the amylose content (LIU, 2005; SHEN et al., 2013). Our group previously demonstrated that arracacha amylopectin can partially react with iodine in apparent amylose analysis (CASTANHA et al., 2018). On the other hand, the presence of short-chain length amyloses can underestimate the apparent amylose content, once the color and intensity of the iodine complex depend on the chain length of amylose - long-sized amylose presents a higher affinity to form an iodine complex than amylose with short size (LIU, 2005; SHANNON; GARWOOD; BOYER, 2009; BERTOFT, 2018). Therefore, the presence of short-sized amylose can underestimate the amylose content, because the formation of the molecule - iodine complex is reduced (SHANNON; GARWOOD; BOYER, 2009). In this way, the ozonation reduced the size of the starch molecules, resulting in molecules with a lower affinity to form an iodine complex, which explains the observed reduction in the apparent amylose content. Thus, the reduction of the apparent amylose content with the ozonation process (Figure 3-D) is related with the described depolymerization (Figure 3-C).

An increase in reducing sugar content with the increasing ozone time was also noticed. The formation of new reducing sugar groups can be associated with the hydrolysis of the glycosidic linkages of starch molecules caused by the ozonation process.

These findings are in accordance with the depolymerization of ozonated potato starches molecules reported by Castanha, Matta junior, & Augusto (2017). On the other hand, Matta Junior et al. (2019), observed slight hydrolysis of the ozone processed cassava starch molecules, which is related to their mild process conditions.

Summarizing, the ozonation process can induce the formation of carboxyl and carbonyl groups, as well as to cause the hydrolysis of glycosidic linkages, promoting the reduction of the molecular size and the formation of reducing sugar groups. These new molecules interact differently among them, which can change the starch properties – as discussed in the next section.

3.3. Starch properties and possible applications

3.3.1. Pasting Properties

Table 1 and Figure 4 exhibit the pasting properties of native and ozonated arracacha starches.

Table 1. RVA parameters of the native and ozonated arracacha starch samples. Peak Apparent Viscosity (PAV), Trough Apparent Viscosity (TAV), Relative Breakdown (RBD), Final Apparent Viscosity (FAV), Relative Setback (RSB) and Pasting Temperature (PT). Average \pm standard deviations; variations followed by the same small letters within a column do not differ significantly (p < 0.05).

| Ozonation Time (min) | PAV (mPa.s) | TAV (mPa.s) | RBD (%) | FAV (mPa.s) | RSB (%) | PT (ºC) |
|-------------------------|---------------|----------------|------------|--------------|------------|------------|
| 0 | 10716 ± 108 a | 2059 ± 18 a | 81 ± 0.3 c | 3111 ± 122 a | 51 ± 7 c | 58 ± 0.1 c |
| 15 | 6633 ± 134 b | 143 ± 9 b | 98 ± 0.1 b | 257 ± 19 b | 79±3a | 59 ± 0.4 b |
| 30 | 448 ± 81 c | 48 ± 2 c | 99 ± 0.0 a | 79 ± 2 c | 65 ± 2 b | 60 ± 0.2 a |



Figure 4. RVA curves of native and ozonated arracacha starch.

The pasting properties of arracacha starch were affected by the ozonation process. The ozonated samples presented a significant peak apparent viscosity (PAV) reduction when compared with the native starch (Table 1, Figure 4). Thus, the PAV decrease indicates a weakening of starch granules, which leads to a minor capacity to maintain the granule integrity.

These results are similar to the findings of other authors (ÇATAL; İBANOĞLU, 2012; ÇATAL; İBANOĞLU, 2014; CASTANHA; MATTA JUNIOR; AUGUSTO, 2017), and they related the weakening of starch granules to the partial cleavage of the glycosidic bonds due to the ozone treatment, which is in accordance with the results of this work.

The ozonation process increased the pasting temperature (PT). These findings are in accordance with Castanha, Matta Junior, & Augusto (2017), and the authors related this behavior with the greatest presence of small-size molecules in the ozonated samples (section 3.2), which require more energy to gelatinize than largesize molecules. According to Çatal & İbanoğlu (2012), small starch molecules have higher gelatinization temperatures, due to possible reassociation of these molecules upon heating. Therefore, this may require a higher temperature to disarrange the resulting reassociation. Moreover, the PT increase could be related to the slightly increase of the size of bigger particles (observed in section 3.1): these bigger granules possibly required higher energy to onset the starch gelatinization and/or the bigger granules can be a consequence of structural changes that can also affect pasting. Even so, we highlight again the changes on granule size was negligible. However, some authors (CHAN; BHAT; KARIM, 2009; OLADEBEYE et al., 2013; ÇATAL; IBANOĞLU, 2014) reported a PT reduction with the increase of ozonation time. On the other hand, Klein et al. (2014) noticed that the ozonation process did not change the PT. These results found in literature can be associated with the different process conditions.

The ozonated starches exhibited an increase in the relative breakdown (RBD – Table 1), which means the granules are more fragile with processing time, being more susceptible to rupture than the native starch. It can be associated with ozonation interference in the intermolecular association ability of polymers, this is possibly due to the partial cleavage of the glycosidic bonds, the reduction of hydroxyl groups and the consequent formation of carbonyl and carboxyl groups in the molecules.

The relative setback (RSB) increased with ozonation. These results indicate that the ozonated samples were more susceptible to re-associate than the native starch, due to the small-size molecules, formed during the ozonation process. The potato starch treated by ozone shown the same behavior, as noticed by Castanha, Matta Junior, & Augusto (2017). Chan, Bhat & Karim (2009), Oladebeye et al. (2013), Çatal & Ibanoğlu (2014), Oladebeye et al. (2018), and Klein et al. (2014) noticed a reduction on the setback values with the increase of oxidation level. However, these authors did not consider the RSB. Therefore, in order to compare the results with the quoted researches, the setback values of this present work are provided by Supplementary Table 1. So, when we considered the setback values (not the RSB) for this present work, they also presented the same tendency of these authors results.

Summarizing, ozone technology was able to change the arracacha starch pasting properties, decreasing the overall apparent viscosity, which can be interesting for some industrial uses. In fact, in the textile industry, the lowest viscosity of starch is interesting for finishes applications in fabrics in order to get penetration and stiffen the cloth (RADLEY, 1976).

3.3.2. Paste clarity and hydrogel texture

The paste clarity of native and ozonated arracacha starch is shown in Figure 5.



Figure 5. Paste clarity of the native and ozonated arracacha starches over 20 days of storage at 5 °C. The triangles, squares and dots are the mean values, the red vertical bars are the standard deviation. The curves are traced to facilitate interpretation.

The native arracacha starch presented a high paste clarity, being in accordance with Castanha et al. (2018) results. According to this author, this high clarity is related to the presence of phosphate groups in the arracacha starch (ranged from 208 to 261 mg kg⁻¹). However, the native starch paste presented highly unstable transparency, since after 20 days of storage, the native paste became opaquer, and there was a ~80% reduction in its clarity. This reduction is possibly related to the retrogradation process: when the starch paste is cooled, the molecules (amylose and amylopectin) rearrange and form a more ordered structure, which causes turbidity effects, hence, reducing the percentage transmittance of the starch paste (WANI et al., 2016; COLUSSI et al., 2017). In this way, the clarity performance of native starch is not interesting for products that demand higher transparency (for example, pie fillings). This information is important to understanding the paste aging, which is rarely conducted in the literature.

On the other hand, the ozonated samples presented clearer pastes if compared to the native starch, as well as a higher stability over storage time - with a high potential for food, paper and textile industrial application. Castanha, Matta Junior, & Augusto (2017), also noticed that ozonated potato starch pastes were clearer than the native ones; however, the authors did not evaluate paste clarity behavior over storage time.

The main factors related to the starch structure that can affect its paste clarity are: The presence of phosphate groups or other electronegative groups (increase paste clarity), and reducing sugar content (increase paste clarity) (CRAIG et al., 1989; VISSER et al., 1997; SITOHY; RAMADAN, 2001). The paste clarity can also be related to granular remnants ("ghosts"/starch fragments – reduces paste clarity) (CRAIG et al., 1989). Moreover, different molecular distribution results in different interactions, including packing effects, which can also affect paste clarity. It is interesting to notice that all the cited factors are changed during ozonation, which difficult point the most important to the observed results.

In native starch, high paste clarity is in general related to the presence of phosphate groups. However, the clearer pastes after ozonation are not related only to these compounds, but with the formation of carboxylic groups (section 3.2): carboxylic groups are electronegatively charged and bulkier than the hydroxyl groups, causing repulsion between the starch molecules. Moreover, the groups of carboxyl hinder the amylose tendency to associate and retrograde, resulting in starch pastes with higher clarity and stability (XIE; LIU; CUI, 2005) – which explains why the processed samples maintain their clarity over storage time. However, the presence of carbonyl groups and different molecular size distribution, make stronger the hydrogels from ozonated starch – as discussed as follows.

In fact, Figure 6 exhibits the native and ozonated arracacha starch hydrogels. As simply demonstrated in this figure, ozonation greatly increased the hydrogel firmness, which is a very interesting result from an industrial perspective. In order to better illustrate the effect of ozonation on the arracacha starch hydrogels, a video demonstrating their consistency is available at https://www.youtube.com/watch?v=guts9wEFDR8&feature=youtu.be.



Figure 6. Native and ozonated arracacha starch hydrogels.

Figure 7 shows three different approaches to evaluate that firmness: the force *versus* penetration depth graphic, before gel disruption (Figure 7-A); maximum force to initiate the starch gels' disruption (Figure 7-B); and, in Figure 7-C, the required energy until reaching the penetration depth of 5 mm (calculated as the area below the curve of "force *versus* penetration depth", and considering 5 mm in order to be the same for all the treatments).



Figure 7. Gel firmness of native and ozonated arracacha starch: (A) force versus penetration depth, (B) maximum peak force, and (C) required energy for a penetration depth until 5 mm. Red bars indicate standard deviations. Variations followed by the same small letters do not differ significantly (p < 0.05).

The native arracacha starch presents a "weak" gel formation when compared with other sources – such as native potato (~23 N) and corn (ranging from 2 to 15 N) starch gel (PRZETACZEK-ROŻNOWSKA, 2017; CASTANHA et al., 2019; DANGI; YADAV; YADAV, 2019). For example, when the native arracacha starch gel is withdrawn from the mold, it is not able to maintain its shape, as illustrated in Figure 6. In order to explain the native starch gel behavior, some aspects of gel formation must be taken into account.

The gel formation is a process characterized by the reassociation of the starch molecules and the development of three-dimensional network structures during storage at low temperatures (BILIADERIS, 2009). According to Ai & Jane (2015), native starches with high amylose content tend to form a stronger gel. However, amylose content cannot be considered the only determinant factor of the final strength of a gel, once it is also related to starch molecular size and gelation conditions (MORRIS, 1990; BILIADERIS, 2009). As reported by Biliaderis (2009), big-sized amyloses make a poor matching and re-association to form a three-dimensional network structure of a gel. On the other hand, amyloses with a short-sized favor the formation of a good network, resulting in gels with higher strength (CHAROENKUL et al., 2011). Also, the amylopectin size influences the rigidity of a starch gel - once a better formation of a three-dimensional network structure is largely related to chain reordering and crystallization of the short-size of this polymer (BILIADERIS, 2009).

Therefore, the behavior of the native and ozonated arracacha starch gels can be related to the size of the molecules and chemical affinity. Native arracacha starches showed molecules with larger size, which impaired a better re-association, resulting in a weak gel formation. On the other hand, the ozonated starches presented a different molecular size distribution, favoring interaction and packing. Moreover, the presence of carbonyl groups possibly induces attractive interaction between carbonyl-carbonyl and carbonyl-hydroxyl groups, which reinforce the new network.

The ozonated samples kept the form of the mold in which they were formed (Figure 6), being this a remarkable characteristic for food industrial use. In fact, the ozonated starches hydrogels required higher forces for disruption than the native starch (Figure 7-A). The ozonated hydrogels exhibited a disruption before reaching a penetration depth of 10 mm, indicating they are more plastics and stronger. In fact, the 30 min ozonated starch gel was 250% harder than the native one (Figures 7-B and 7-C).

Observing the results of the ozonated samples (higher strength when compared to native starch gel), they seem to be contradictory, since the presence of carboxylic groups on the processed starches is in general associated with a weaker gel, once this group is related to the repulsion between the starch molecules – which increased the clarity of the ozonated starch pastes. However, some points must be taken into account, in order to understand these findings.

First, the ozonation process changed the molecular size distribution (reduction of bigger-size molecules and increase of the smaller ones), which greatly affects the interaction among them – once small-size molecules, as mentioned before, favors the formation of a good network structure of a gel, which improves its final strength. Second, although carboxylic groups are associated with repulsion between starch molecules, the ozonation also produced carbonyl groups. In fact, carbonyl groups form strong hydrogen bonds with the hydroxyl groups (ZHANG et al., 2013) and maybe with carbonyl-carbonyl. All these effects vary together during ozonation. Consequently, the final interaction and network formation is a complex relation among these effects.

In summary, the ozonated arracacha starch presented harder gels than the native sample, which is promising for industrial use, once stronger gels are desired for products such as desserts (puddings and flans), jelly candies and meat products (sausages). Moreover, starch gels with greater firmness have aroused interest in the application of 3D printing of food, presenting better printability (MANIGLIA et al., 2020).

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

Figure 8 presents the WSI (Figure 8-A) and WAI (Figure 8-B) results for the native and modified arracacha starches.



Figure 8. (A) Water solubility and (B) water absorption indexes of the native and ozonated arracacha starches at different temperatures. The dots are the mean values, the vertical bars are the standard deviation. The curves are traced to facilitate interpretation.

As the ozonation time and the gelatinization temperature increased, higher values of WSI (Figure 8-A) were observed, being more significant at 60 °C. From an industrial point of view, a higher WSI is sometimes desirable.

Castanha et al. (2018) observed that the gelatinization temperature of the arracacha starch is low (~55 °C) in comparison with other sources. In this way, during starch gelatinization, when it is subjected to heating in the presence of water, the granules absorb water and swell, and the starch molecules – mainly amylose – leach out and solubilize (MOORTHY; SAJEEV; ANISH, 2018). Therefore, the higher solubility on the ozonated samples can be associated with the depolymerization (section 3.2), since the small-sized molecules easily leach.

Chan, Bhat, & Karim (2009), and Oladebeye et al. (2013) also noticed an increase of WSI for the ozonated starches and they associated their results to a structural weakening of the starch granules and the depolymerization. Moreover, Castanha et al. (2019) related this increase of the WSI in the modified starch due to the formation of more electronegative groups and molecular cleavage.

The ozonated samples presented a slight increase in the water absorption index (WAI) at the temperatures of 45 and 50 °C when compared with the native starch. On the other hand, at higher temperatures (55 and 60 °C), with the increase of the ozonation time, the ozonated starch samples presented lower WAI than the native starch. Some authors (CHAN; BHAT; KARIM, 2009; OLADEBEYE et al., 2013; CASTANHA et al., 2019) also observed a WAI reduction with increasing ozonation

time. Chan, Bhat, & Karim (2009), and Oladebeye et al. (2013) related their results to a structural disintegration within the starch granule during the modification process.

In summary, the ozonation process promoted an increase in the WSI and reduction of WAI, which can be useful in some industrial applications.

4. CONCLUSIONS

This work evaluated the ozonation process of arracacha starch for the first time.

The ozone technology was able to affect the arracacha starch molecules, such as follows:

- Formation of carbonyl and carboxyl groups due oxidation of the starch hydroxyl groups;
- Reduction of the pH of the ozonated starch suspensions the formation of the carboxyl group increased the acidity of the samples;
- Reduction of bigger molecules and increase of the smaller ones caused by molecular depolymerization;
- Decrease of apparent amylose content once the ozonation treatments reduced the size of the molecules, the molecules presented a lower affinity to form an iodine complex, leading to a reduction in the apparent amylose content;
- Increase in reducing sugar content the ozonation process promoted hydrolysis in the glycosidic linkages of starch molecules.

Due to these molecular changes, the ozonation process affected arracacha starch properties, such as: pasting properties (reduction on its viscosity), paste clarity (clearer pastes with stability over storage time), hydrogels texture (when compared with the native starch gel), water absorption (reduced) and water solubility (increased) indexes. In this way, most of the arracacha starch properties changes can benefit its industrial application. Therefore, the ozonation process promoted changes in arracacha starch properties, enabling its better industrial use.

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LIMA, D. C.; MANIGLIA, B. C.; MATTA JUNIOR, M. D.; LE-BAIL, P.; LE-BAIL, A.; AUGUSTO, P. E. D. Dual-process of starch modification: combining ozone and dry heating treatments to modify cassava starch structure and functionality. **International Journal of Biological Macromolecules**, in press, 2020.

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ABSTRACT

This work evaluated for the first time the effect of dual modification of cassava starch by using ozone (O₃) and dry heating treatment (DHT). The dual modification was capable to promote fissures on the surface of the starch granule (DHT+O₃), affected the starch amorphous domains, presented greater degree of starch oxidation (DHT+O₃) and different profiles of starch molecular size distribution. These modifications resulted in starches with different properties. Moreover, the sequence of treatments was decisive for the hydrogel properties: while DHT+O₃ resulted in formation of stronger gels, O₃+DHT resulted in weaker gels. In conclusion, this proposed dual modification was capable to produce specific modified starch when compared with the isolated treatments, also expanding the potential of cassava starch applications.

Keywords: starch modification, chemical modification, DHT.

1. INTRODUCTION

Starch is composed by two polysaccharides (amylose and amylopectin), as well as traces of proteins, fats, minerals, fibers, and water, organized in microparticles with both crystalline and amorphous regions called granules. In nature, starch is used as energy storage in plants, being found in different botanical sources (mainly cereals, pulses, roots, and tubers). From an economic point of view, starch is an important natural biodegradable ingredient for different industries, such as food, feed, cosmetic, chemical, pharmaceutical, paper, textile, petrochemical, among others. In food industry, starches are widely applied for thickening, texturizing, gelling, and stabilizing (ALTUNA; HERRERA; FORESTI, 2018). However, native starch has limited properties, which impairs its industrial application (ASHOGBON; AKINTAYO, 2014; ZIA-UD-DIN; XIONG; FEI, 2017) - for instance, considering cassava starch, its weak gel can be pointed as an industrial limitation. Therefore, industries have been using different processes to modify and enhance starch functional properties.

The main starch modification technologies are: physical, chemical, enzymatic, and genetic. Chemical modifications (acetylation, oxidation, cross-linking, hydroxypropylation and etherification) are extensively used by the industry to modify native starches. However, it can be hazardous to the consumers and/or environment (QIU et al., 2015; VANIER et al., 2017), or even limit applications (such as in food and

pharmaceutical fields). Moreover, recently, consumer and industry preferences have focused on "environmentally friendly" modification technology and sustainable products (MANIGLIA et al., 2020b).

Among these methods, ozone technology is considered an "environmentally friendly" chemical treatment for modifying starch, once ozone decomposes into oxygen without leaving toxic by-products (ÇATAL; İBANOĞLU, 2014; PANDISELVAM et al., 2019b; MANIGLIA et al., 2021).

Ozone as a starch modification technology has already been studied for different starch sources. Most of these studies observed that ozone processing has caused significant changes in the structure of starch as reduction of the size of amylose and amylopectin, and also replacement of the hydroxyl groups by carbonyl and carboxyl groups (through oxidation) (HANDARINI et al., 2020a). In this way, ozone technology has been considered as a promising technology for starch modification once it has promoted improvements in starch functional properties. Considering cassava starch, ozone processing resulted in clearer pastes, stronger hydrogels even in acidic conditions (LIMA et al., 2020a), enhanced oven expansion and biscuits texture (MATTA JUNIOR et al., 2019), enhanced biodegradable plastics (LA FUENTE et al., 2019), and 3D printed gels (MANIGLIA et al., 2019).

Another interesting "environmentally friendly" method is the dry heat / dry heating treatment (DHT), a physical modification method that does not use chemical reagents and only heat, thus being also considered the environment friendly (MANIGLIA et al., 2020b, 2021).

In fact, this modification technology has been evaluated on different starch sources (SUN et al., 2013, 2014; PRAMODRAO; RIAR, 2014; QIU et al., 2015; CHANDANASREE; GUL; RIAR, 2016; JI et al., 2016; OH; BAE; LEE, 2018; LIU et al., 2019; MANIGLIA et al., 2020a, 2020c). In the literature, it was observed that DHT can modify the physicochemical properties of starch without destroying its granule structure (SUN et al., 2014), and it can also promote oxidation and depolymerization of the starch polymers resulting in cassava starch hydrogels with better printability in 3D printing (MANIGLIA et al., 2020a).

Even though ozone treatment and DHT are good alternatives for modifying starch, there is a trend to evaluate combinations of treatments, in order to promote new types of starch modification and bring new features (CASTANHA et al., 2019; HU et

al., 2019; LI et al., 2020; OMODUNBI ASHOGBON, 2020). The interaction between different technologies can potentialize the results and the possible results are unpredictable. We hypothesized the combination of ozone and dry heating treatment, as well as its sequence, can promote new types of starch modification when compared to the isolated treatments.

In this context, the combination of ozone and dry heating treatments to promote starch modification has not yet been investigated. Consequently, this work studied for the first time the combination of ozone and dry heating treatments to modify the structure and functionality of cassava starch.

2. MATERIAL AND METHODS

2.1. Raw material, ozone (O_3) and dry heating treatments (DHT), and combination of the processes (O_3 +DHT, DHT+ O_3)

The native cassava starch used in this study was a regular commercial product (Amilogill 1500) kindly provided by Cargill Agrícola S.A. (Campinas, SP, Brazil).

Ozone and dry heating treatments (DHT) were conducted considering previous conditions that resulted in stronger gels (LIMA et al., 2020a; MANIGLIA et al., 2020a).

The ozone treatment was performed using a similar system described by Lima et al. (2020a), and it is illustrated in Figure 1. In short, the ozone was produced in an ozone generator unit (Ozone & Life, O&G Model RM 3.0, São José dos Campos, Brazil) by the coronal-discharge method from industrial oxygen (95% purity). The sample (10 g dry starch/100 g deionized water suspension, 1500 mL) was placed in a glass reactor (2 L capacity, 56 cm of height, 6 cm of internal diameter) and processed for 30 minutes, being constantly stirred with a magnetic stirrer (300 rpm). The gas flow in the reactor was maintained constant at 1 L min⁻¹, and the ozone concentration was measured by an ozone monitor (2B 1Technologies Model 106-H, USA), being its concentration kept at 45 mg O₃ L⁻¹. After each ozone treatment, the suspensions were maintained at rest for one hour to promote decantation, and the supernatant was discarded. The starch was dried in an air circulation oven at 35 °C until ~12% of moisture content (in wet basis). The dried samples were then milled in a mortar, sieved in a 60-mesh sieve (250 μ m) and placed in glass flasks for further analysis.



Ozone Treatment (O₃)

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Figure 1. Schematic representation of ozone and dry heating treatments (DHT).

The dry heating treatment (DHT) process was carried out as proposed by Maniglia et al. (2020a) (Figure 1). The starch samples (50 g) were distributed evenly in a thin layer (~ 1 mm) and packaged on aluminum foil (30 x 30 cm, width x height), and closed with tape to ensure no loss of material. Then, the samples were placed in a hot-air convective oven (MA 035, Marconi, São Paulo, Brazil) at 130 °C for 4 h (air velocity $1 \pm 0.1 \text{ m s}^{-1}$). After the modification process, the starch package was cooled at room temperature, then the samples were sieved in a 60-mesh sieve (250 µm) and stored in glass flasks for further analysis.

Two approaches were applied regarding the combination of both technologies: I.The ozone treatment was performed first. Then the dry heating treatment process were carried out on the ozonated samples. The samples that pass through these treatments were named as "O₃+DHT". II. The dry heating treatment process was conducted first, then the ozone treatment was performed on dry heating treated samples. The starch samples that were modified following this approach were named as "DHT+O₃".

Native, ozonated (O_3) , and dry heating treated (DHT) starches samples (without the combination of both treatments) were used as control.

2.2. Starch granule characterization

2.2.1. Granule morphology

The cassava starch granules morphology was evaluated by light microscopy.

A light microscope (model L1000, Bioval, Curitiba, Brazil), with a 20 W halogen lamp, was used to observe the starch granules' shape, surface and edges. The samples (0.1 g) were dispersed in 10 mL of distilled water with 50 μ L of Lugol. Then a drop of this solution was placed on a glass slide covered by a glass cover slip. The magnification used to obtain the images was 400x. To observe the Maltese crosses, a polarized light filter was coupled to the system.

2.2.2. Granule relative crystallinity

In order to evaluate de relative crystallinity, it was used an X-ray diffractometer (Shimadzu XRD 7000, Tokyo, Japan) with Cu radiation, using the following conditions: angle 20 ranging from 3 to 50 °, scan rate of 2 °·min⁻¹ and a voltage of 30 kV and amperage of 15 mA. The starch samples were maintained in a desiccator containing saturated BaCl₂ solution (25 °C, aw = 0.90) for 10 days to equilibrate the moisture content. The relative crystallinity was calculated as described by Nara and Komiya (1983), considering 20 ranging from 3 to 30 °, and using the Origin software version 9.6.5 (Microcal Inc., Northampton, MA, USA).

2.3. Starch molecular characterization

2.3.1. pH

The pH was evaluated using a calibrated potentiometer (Tecnal, TEC-5 mode, Piracicaba, Brazil). The starch samples (2 g, in dry basis) were suspended in 20 mL distilled water, being constantly stirred with a magnetic stirrer (100 rpm) (POLESI et al., 2016).

2.3.2. Carbonyl and carboxyl groups content

Carboxyl and carbonyl contents were measured as described by Lima et al. (2020a), with some modifications.

To evaluated the carboxyl group content, the starch sample (2 g, in dry basis) was dispersed in 25 mL of HCl solution (0.1 M) and stirred for 30 min (using a magnetic stirrer, 100 rpm) at room temperature (~ 25 °C). Next, the samples were vacuum filtered using qualitative filter paper (205 μ m) and washed with 400 mL of distilled water, in order to remove the residual acid. Then the obtained starch was suspended in 300 mL of distilled water and brought to a boiling water bath with continuous manual stirring for 15 minutes to ensure gelatinization. After this, the hot gelatinized starch was titrated with a NaOH (0.01 M) solution until pH 8.3 (using a burette and a potentiometer Tecnal, TEC-5 mode, Piracicaba, Brazil). The native cassava starch was used as a blank. The result was expressed as the amount of carboxyl groups per 100 glucose units (COOH/100GU), according to Equation 1.

$$\frac{\text{COOH}}{100\text{GU}} = \frac{[(\text{Vs} - \text{Vb}) \cdot \text{M} \cdot 0.045 \cdot 100]}{\text{SM}}$$
(1)

Where:

Vs = volume of NaOH required for the modified samples;

Vb = volume of NaOH used in the blank;

M = molarity of NaOH;

SM = sample mass in dry basis.

The carbonyl group content analysis was performed as follows: 2 g (in dry basis) of starch samples was dispersed in 100 mL of distilled water, then they were placed in a boiling water bath and manual stirred for 20 minutes (until complete gelatinization). The gelatinized sample was cooled at 40 °C and adjusted to pH 3.2 (potentiometer - Tecnal, TEC-5 mode, Piracicaba, Brazil) with a HCI (0.1 M) solution. An aliquot of 15 mL of hydroxylamine solution (5% of hydroxylamine hydrochloride and 20% of 0.5 M NaOH solution in distilled water) were added to the sample. Then, the samples were placed in a 40 °C water bath with stirring for 4 h. Thereafter, the excess of hydroxylamine was titrated with 0.1 HCl, using a burette, until pH 3.2 and to control the pH of the samples (potentiometer - Tecnal, TEC-5 mode, Piracicaba, Brazil). A blank,

without sample was prepared with all reagents. The result was expressed in quantity of carbonyl groups per 100 glucose units (CO/100GU), according to Equation 2.

$$\frac{CO}{100GU} = \frac{[(Vb - Vs) \cdot M \cdot 0.028 \cdot 100]}{SM}$$
(2)

Where:

Vb = volume of HCl used in the blank; Vs = volume of HCl required for the sample; M = molarity of HCl; SM = sample mass in dry basis.

2.3.3. Gel permeation chromatography

The molecular size distribution profile was evaluated by using a gel permeation chromatography (GPC), following the methodology of Song and Jane (2000) with modifications. A GE Healthcare - 28988950 XK 26/70 column (2.6 cm diameter and 70 cm high) packed with Sepharose CL-2B gel (Sigma, Sweden) was used. First, 0.1 g (in dry basis) of starch was dispersed in 10 mL of dimethylsulfoxide (DMSO, 90%, Labsynth, Brazil), then the solution was heated in a boiling water bath for 1 h and kept at 25 °C for 16 h under constant stirring. Then 3 mL of the solution was mixed with 10 mL of absolute ethanol and centrifuged for 30 min at 3000 g. The supernatant was discarded, and 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 fractions of 4 mL of the eluted solution in different tubes. The samples were evaluated for total carbohydrate content (TCH) (at a wavelength of 490 nm) by the phenol sulfuric method (SANDHU; MANTHEY; SIMSEK, 2012) and blue value (BV) method at 620 nm (CASTANHA et al., 2020), using a spectrophotometer (spectrometer Femto, Model 600S, São Paulo, Brazil).

2.4. Starch hydrogels strength

In order to measure the gel strength, starch hydrogels were prepared using 10.7% of starch in distilled water. The samples were gelatinized in a boiling water bath

for 15 min under vigorous mechanical agitation, using a mechanical stirrer (250 rpm). Then the gelatinized starches were stored at 5 °C for 24 h in a 40 x 20 mm (diameter x height) plastic cup in a desiccator (with water at the bottom to ensure a uniform moisture). The starch hydrogels strength was determined by a puncture assay using a Texture Analyzer (TA.XT Plus, Stable Micro Systems Ltd., Surrey, UK) with a load cell of 50 kgf (490.3 N). The samples were compressed, with a cylindrical probe (P/0.5R, 12.7 mm of diameter), until the distance of 10 mm at 1 mm s⁻¹.

To illustrate the differences in consistency, we also took pictures of the gels falling from a spoon and the unmold gels on a surface to characterize their spreading (Figure 6). We also evaluated the printability of the gels using a syringe (die diameter of 1.5 mm) and extruding this material on a surface (6 cm lines) for visual assessment of the gels consistency and moldability at room temperature (Figure 6). According to Maniglia et al. (2019), this simple analysis is a good indication of the material printability, presenting a good correlation with real 3D printing.

2.5. Color characterization: starch powder and hydrogel

The color of the native and modified starches, as well as their hydrogels, was evaluated using colorimeter Chroma Meter CR-400 (Konica Minolta Sensing Americas, Inc, Ramsey, USA). A CIE-Lab color scale was used to measure the parameter of lightness L* (L* = 0 black and L* = 100 white), the chromaticity parameters a* (+a* = red and $-a^*$ = green) and b* (+b* = yellow and $-b^*$ = blue) of the samples. The results were expressed as the average of three measurements of the starches powders and their hydrogels. The total color difference (ΔE^*) was calculated using Equation (3): $\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$ (3)

Where ΔL^* , Δa^* , and Δb^* are the differences between the L*, a*, and b* parameters of the modified samples and the native starch.

2.6. Experimental design and statistical analysis

The experimental design was completely randomized. Each treatment was made in duplicate and the samples were analyzed in triplicate. Data were assessed by the analysis of variance (ANOVA) and comparisons of averages for treatments were made using the Tukey test, using the software Minitab version 19 (Minitab, LLC., USA).

3. RESULTS AND DISCUSSION

3.1. Starch granule characterization: Granule morphology and relative crystallinity

Figure 2 shows the microscopy of control and modified cassava starches. Both native and modified cassava starch granules exhibited a round, polygonal, angular and truncated shapes, being in accordance with other works (KLEIN et al., 2014; DUDU et al., 2019; HAN et al., 2019).



Figure 2. Microscopic images of native and modified cassava starches (O₃, DHT, DHT+ O₃, O₃ + DHT). Images from the first column are light microscopy. The second column shows further details (without scale). The third column shows the Maltese crosses of each sample. The black line on the bottom left represents the first and second columns images' scale.

Based on Figure 2, we observed that each treatment alone, as also the O_3 +DHT were not capable to change the morphology of the starch granules nor surface characteristics. Previous works that used ozone or dry heating treatment to modify starch, have also observed that these treatments did not change the surface of the granules (KLEIN et al., 2014; LIMA et al., 2020a; MANIGLIA et al., 2020a). On the other side, the DHT+O₃ modification treatment was able to cause fissures on the surface of the starch granule, as displayed in the second column of Figure 2. Even though the DHT alone did not change the surface of starch granules, this process possibly weakened the surface structure, making it more susceptible to cracking with the next treatment (ozone). However, this behavior was not observed in the inverse sequence of treatments (ozone and later DHT).

For all modified starches, no changes were observed in the Maltese crosses (Figure 2), which indicates that the modification processes did not significantly affect the internal microstructure of cassava starch granules.

The X-ray diffraction patterns of starch samples and their respective relative crystallinity (RC) are displayed in Figure 3. Table 1 shows the Bragg angles, Miller indexes, and interplanar spacings (d) for the native and modified starches. The identification of each peak was based on the PDF 43-1518 reported in the literature (IMBERTY et al., 1988).



Figure 3. (A) X-ray diffraction pattern and (B) relative crystallinity (%) of starch samples (native, O₃, DHT, DHT+O₃, and O₃+DHT). Red bars indicate standard deviations. Variations followed by the same small letters do not differ significantly (p < 0.05).

| Bragg angle (2θ) | Miller indexes (<i>hkl</i>) | d (Å) |
|------------------|-------------------------------|-------|
| 11.20 | 101 | 7.94 |
| 15.27 | 121 | 5.85 |
| 17.20 | 031 | 5.21 |
| 18.09 | 211 | 4.97 |
| 20.40 | 040 | 4.42 |
| 23.30 | 310 | 3.90 |
| 26.67 | 051 | 3.43 |
| 30.70 | 400 | 3.02 |
| 33.30 | 143 | 2.81 |
| 38.25 | 352 | 2.49 |

Table 1. Bragg angles, Miller indexes, and interplanar spacing (d) for the native and modified starches.

In Figure 3-A, we observed that all starch powder exhibited the same peaks and it is a typical A-type starch pattern. In addition, as presented in Table 1, it is possible to observe the cassava starch crystalline structure corresponds to an orthorhombic phase (IMBERTY et al., 1988), which is similar to that one showed by Rodriguez-Garcia et al. (2020) for amaranth starch. Moreover, the modified starches maintained the same X-ray diffraction pattern of the native cassava starch, as also the relative crystallinity (RC) that did not vary significantly (p < 0.05) (Figure 3-B), which indicates that all modification processes did not change the crystalline orthorhombic phase of starch.

Castanha, Matta Junior, and Augusto (2017) also noticed that potato starch processed by ozone did not present any significant change in the RC when compared with the native starch. Furthermore, Handarini et al. (2020b) evaluated arrowroot starch processed by gaseous ozone system and also observed that XRD patterns remained unchanged. The authors stated the ozonation did not affect the crystalline domain. However, they observed a reduction in the RC of the modified starches. Possibly this reduction is related to the extent of modification in their work once the authors used a different ozonation system and starch source. Other works, such as Klein et al. (2014), and Lima et al. (2020a), reported that ozone treatment slightly reduced the RC in cassava starches. According to these authors, this reduction demonstrates the ozone acts not only in the amorphous lamellae, but also in the starch semi-crystalline domain. Oh et al. (2018), Liu et al. (2019), Zhou et al. (2019), and

Maniglia et al. (2020a) evaluated the effects of DHT in proso millet, high amylose rice, rice, and cassava starches, respectively, and they reported a reduction in the RC.

On the other side, in this present work, we observed that RC did not vary significantly (p <0.05) between the native and the modified starches (Figure 3-B). Moreover, the similarities in the X-ray diffraction patterns, observed in this work, indicate the processes (either alone or in combination) did not alter the long-range crystalline structure (crystalline domain). This indicates the modifications may be affecting mainly the amorphous zones with little change in the crystalline region, similar to that pointed out by Xie, Liu, and Cui (2005) on the oxidation behavior in starches. In fact, it is important to highlight the previous reported changes on relative RC, when observed, are always very small, reinforcing the most important changes due to both ozone and DHT treatments take place in the amorphous region. In addition, the literature have shown that changes in the starch amorphous domain affects the functionality of starch (ELIASSON; GUDMUNDSSON, 2006; CHEVIGNY et al., 2018). It worth highlighting the amorphous domain is composed of several materials, for instance, free amylose, lipid-complexed amylose, and mainly branching points/areas of the amylopectin (ELIASSON: GUDMUNDSSON, 2006; HIZUKURI; ABE; HANASHIRO, 2006; JANE, 2009). Therefore, we hypothesized the changes were promoted in amylose and in the branching points of amylopectin, which contributed with the alteration in the short-range order in the modified starches. In fact, it was observed molecular depolymerization in the molecules of the modified starches (section 3.2), which confirms the modification processes affected the amorphous zones of the starch.

Summarizing, even though the granule internal microstructure and the relative crystallinity of the modified starches did not present big changes, it is important to highlight that each modification treatment was able to modify the starch molecular structure, as presented in section 3.2.

3.2. Starch molecular characterization: pH, carbonyl and carboxyl contents, molecular size distribution

The starch molecular modification performance can be evaluated by the changes on molecular length and degree of oxidation. The degree of oxidation can be evaluated by the carbonyl and carboxyl groups, as well as their sum, according to Barbosa et al. (2019). This evaluation is shown in the Table 2. Moreover, the

depolymerization promoted by cleavage of glycosidic bonds can be evaluated by the molecular size distribution, as shown in Figure 4.

| Starch samples | рН | Carbonyl | Carboxyl | Degree of |
|-----------------------|--------------------------|-------------------------|-------------------------|--------------------------|
| | | content | content | oxidation |
| | | (CO/100 GU) | (COOH/100 GU) | (CO + COOH) |
| Native | 5.75 ± 0.15 ^a | $0.00 \pm 0.01^{\circ}$ | * | 0.00 ± 0.01^{d} |
| O ₃ | 3.96 ± 0.08^{b} | 0.08 ± 0.01^{b} | 0.03 ± 0.00^{b} | 0.11 ± 0.01^{b} |
| DHT | 5.49 ± 0.13^{a} | 0.06 ± 0.01^{b} | $0.00 \pm 0.00^{\circ}$ | 0.06 ± 0.01 ^c |
| DHT + O ₃ | 3.85 ± 0.07^{b} | 0.13 ± 0.01^{a} | 0.04 ± 0.00^{a} | 0.17 ± 0.01 ^a |
| O ₃ + DHT | 4.06 ± 0.07^{b} | 0.09 ± 0.01^{b} | 0.03 ± 0.00^{b} | 0.12 ± 0.01^{b} |

Table 2. pH, carbonyl and carboxyl contents of native and modified cassava starches.

Average \pm standard deviations; variations followed by the same letters do not differ significantly (p < 0.05). *Carboxyl content is considerate zero since native starches do not have this functional group.



Figure 4. Molecular size distribution: (A) blue value (BV) and (B) total carbohydrates (TCH) results of the gel permeation chromatography (GPC) analysis for native and modified starches.

The molecular size distribution was obtained by GPC analysis, which is a sizeexclusion technique that consists of the separation of the polysaccharide molecules according to their molecular size (HIZUKURI; ABE; HANASHIRO, 2006). Starch polysaccharide molecules are placed into a column filled with a gel with a specific porosity: bigger molecules elute first, whereas smaller ones are retained in the gel pores and elute later (BRUMMER; CUI, 2005; HIZUKURI; ABE; HANASHIRO, 2006). In this method, the bigger molecules are the firsts fractions, can be associated with amylopectin molecules (first peak), while the smaller ones elute latter and are related to amylose molecules (second peak). After separation, the fractions were evaluated by both the blue value (BV) and the total carbohydrate (TCH) methods, as presented on Figure 4.

According to Wurzburg (1986), the oxidation affects the hydroxyl groups at C-2, C-3, and C-6 positions of the starch molecules, forming carbonyl and carboxyl groups. Simultaneously, the chain scission (depolymerization) might occur in severe oxidation processes, as ozone processing, reducing the size of the starch molecules. Usually, the depolymerization occurs by the cleavage of glycosidic bonds, mainly α -(1,4)-bonds, leading to debranching of amylopectin chains and shortening of amylose chains (KURDZIEL et al., 2019).

In this way, we can observe that the high oxidant power of ozone resulted in the formation of both carbonyl and carboxyl groups for the O₃, DHT+O₃, and O₃+DHT treatments (Table 2). Allied to that, as demonstrated in Figure 4, the ozone process was the treatment which promoted more significant depolymerization of starch molecules: the treatments with ozone presented significant cleavage of large-size molecules (first peak practically disappeared) and increase in the fraction of small-size molecules (displacement to the right, narrowing and increasing intensity of the second peak). This behavior is in accordance with the total carbohydrates profile (Figure 4-B) and previous works (CASTANHA; MATTA JUNIOR; AUGUSTO, 2017; MANIGLIA et al., 2019; CASTANHA et al., 2020; LIMA et al., 2020b, 2020a). Therefore, the ozone treatment was the process, which more contributed for the starch molecular modification performance (higher degree of oxidation and greater reduction of molecular-size).

DHT resulted in formation of only carbonyl groups in the starch molecules (Table 2). In fact, the atmospheric oxygen can initiate oxidation to a lesser extent during thermal treatment, leading to the formation of carbonyl groups (KURDZIEL et al., 2019). However, the oxidation was not strong enough to produce carboxyl groups. In Figure 4, regarding the size molecular distribution, we can observe that DHT reduced the fraction of molecules with intermediate size with consequent increase in the second peak, which was also displaced to the right (increase of small-size molecules fraction). Maniglia et al. (2020a) and Nunes et al. (2016) also observed that DHT can promote depolymerization in the starch molecules. Compared to ozone treatment, DHT was a process, which contributed less for starch modification performance (lower degree of oxidation and lower reduction of molecular-size).

In relation to the combined treatments (DHT+O₃ and O₃+DHT), as mentioned, ozone process presented the greatest contribution to molecular changes, once these starches exhibited the presence of carboxyl groups and a similar molecular-size distribution profile of O₃ starch. However, the combination sequence of first DHT and later O₃ (DHT+O₃) resulted in greater degree of oxidation than the other treatments. Possibly, the DHT increased the availability of sites to undergo oxidation what allowed O₃ treatment to promote oxidation in more sites, resulting in more content of functional groups in this modified starch. In fact, according to Klanarong et al. (2020), the starch depolymerization increases the amount of available sites to undergo oxidation, resulting in formation of new functional groups. On the other hand, the inverse sequence (O₃+DHT) presented a different behavior: the oxidation degree was similar to the O₃ starch. This behavior can be related with the high oxidant power of ozone, which possibly reacted with most of the available sites to undergo oxidation, reducing the possibility of DHT in forming more carbonyl and carboxyl groups.

Finally, as expected, the presence of carboxylic groups decreases the starch suspensions pH (O₃, DHT+O₃, and O₃+DHT) when compared to the native starch (Table 2).

Regarding the BV results, O₃, O₃+DHT, and DHT+O₃ starches showed similar molecular size distribution, with reduction of the larger-size molecules fraction and a significant increase of the small-size molecules fraction, when compared to the native starch. It was also observed a slight difference in the fraction of small-size molecules of the O₃ starch, which showed an enlargement of the second peak, while the O₃+DHT and DHT+O₃ starches showed a narrowing. Before explaining this behavior, it important to highlight that BV analysis is based on the capacity of the starch molecules to form an iodine complex, resulting in the BV profile. Thus, according to the literature (LIU, 2005; SHANNON; GARWOOD; BOYER, 2009; BERTOFT, 2018), big-sized molecules presents a higher tendency to form an iodine complex than the small-sized ones. In this way, we suppose the combination of treatments reduced the size of the molecules to a too-small size that they did not favor the iodine complex, resulting in the narrowing of the second peak fraction. In addition, DHT+O₃ showed a more significant fraction of intermediate-sized molecules than O₃+DHT, and it can be confirmed by TCH results (Figure 4-B). Possibly, the reduction of intermediate-sized molecules in the O₃+DHT starch is related to the great oxidant power of the ozone treatment, because in the first treatment (O₃) of the combination, the larger-size fraction already was totally cleavage, resulting in a molecular-size profile already very reduced.

Summarizing, the ozone treatment was essential for the carboxyl groups formation and reduction of the molecular size. In addition, the dual combination can result in starches with different molecular profile (size and degree of oxidation) in relation to the native and modified starches obtained using a single treatment. Moreover, the sequence of the treatments (ozone and dry heating treatment) influenced in the degree of oxidation, promoting differences in the content of carbonyl groups and in the molecular size distribution profile (depolymerization). The consequent starch properties must then be evaluated.

3.3. Starch properties and possible applications

Figure 5 presents images of the starch powder and their hydrogels, as well as their color parameters (L*, a*, and b*) and differences (ΔE^*) in relation to the native starch.



Figure 5. Images and color parameters (L*, a*, b*, and ΔE^*) of the native and modified cassava starch powder and hydrogels. Red bars indicate standard deviations. Variations followed by the same small letters do not differ significantly (p < 0.05).

All modification processes changed the color of starch powder and hydrogels, although slightly. The O₃+DHT starch powder exhibited the greatest color difference (ΔE^*) when compared to the other samples, and more yellowish shade (+b^{*}) and

darker (L*) than the other starches. The O₃ and DHT+O₃ starches powders, as also their hydrogels, were lighter than native, O₃+DHT, and DHT starches. The whiteness of the O₃ and DHT+O₃ starch powder and their hydrogels possibly may be related to the ozone process capacity of bleaching and causing discoloration in foods, as reported in literature (TIWARI et al., 2008; PANDISELVAM et al., 2019a). The darkening of the DHT and O₃+DHT powder may be related to the heating process: color formation by heating in the absence of nitrogenous compounds is the result of the caramelization process (HOBBS, 2009).

Hydrogels based on modified cassava starches showed greater luminosity (mainly O₃, DHT, and DHT+O₃) and lower red shade (a^{*}) and blue shade (b^{*}) than the hydrogels based on native starches. The highest values of ΔE^* and luminosity were found in the O₃, and DHT+O₃ starch hydrogels, and possibly it is related to the starch powder bleaching, which reflected in the hydrogels' luminosity. In the case of O₃+DHT hydrogels, the color of this gel has the contribution of the caramelization process of the starch.

Summarizing, we can conclude that the last process used in the combined treatments is decisive for the parameter colors of the starch powder (for instance, if ozone treatment was the last one, it results in a whiter starch powder, and if DHT was the last one, it results in a darker starch powder). Also, we observed that the dual combination, the treatment O_3 and later DHT, resulted in starches more colored and their hydrogels less colored than native and the other modified starches.

Regarding the texture of the hydrogels, Figure 6 displays images of the hydrogels produced in this work, representing their consistency and potential for 3D-print food application (the gel line in front of the unmolded hydrogels). Furthermore, Figure 7 exhibits the hydrogel strength of native and modified cassava starches by using three approaches: force (N) *versus* penetration depth (mm) profile of the starch gels (Figure 7-A), maximum force to initiate the disruption of the starch gels (Figure 7-C) (calculated as the area below the curve of "force *versus* penetration depth").

Consistency of starch hydrogels



Starch hydrogels after being removed from the mold



Figure 6. Images of native and modified cassava starch hydrogels representing the consistency (gels falling from a spoon), their behavior when unmolded (surface spreading), and their printability (lines produced using a syringe).



Figure 7. Hydrogel strength of native and modified cassava starches: (A) Force versus penetration depth profile, (B) maximum peak force, and (C) the total energy needed to reach the penetration depth of 10 mm. Red bars indicate standard deviations. Variations followed by the same small letters do not differ significantly (p < 0.05).

The modified cassava starches by the O_3 , DHT, and DHT+ O_3 treatments developed stronger gels than the native form (Figure 7), and they were able to keep their shape after being withdrawn from the mold (Figure 6). Also, these hydrogels (O_3 , DHT, and DHT+ O_3) start to break before reaching the preset penetration depth of 10

mm (Figure 7-A), evidencing that they presented a plastic texture – Figure 6 illustrates this behavior.

In Figure 7-A and B, it was observed that the hydrogels based on DHT and DHT+O₃ starches required a higher force than the other hydrogels to start to disrupt their gels, being ~ 660% stronger than the native starch gel. However, even though the hydrogels based on DHT and DHT+O₃ starches presented similar strength to initiate the disruption of their gels (Figure 7-B), the DHT+O₃ hydrogel started to break before the DHT one. Moreover, as the probe penetrated the DHT+O₃ hydrogel (Figure 7-A), higher forces were required to further break its gel, whereas in the DHT hydrogel, a continuous reduction in the breaking strength of the gel was observed. Additionally, in Figure 7-C, one noticed that the hydrogel based on DHT+O₃ starch presented the highest energy to puncture 10 mm of the gel. It indicates that the hydrogel based on DHT+O₃ was more resistant than the others.

The hydrogels potential for 3D printing was evaluated as mentioned by Maniglia et al. (2019): the hydrogels were extruded using a syringe to observe the behavior of the line deposited on a glass surface (mainly how this hydrogel spreads, which can be correlated with the hydrogel strength). This analysis is a good indication of the printability of the hydrogel and has a good correlation with its behavior when used in real 3D printing. In this way, the stronger hydrogels (DHT, O₃, and DHT+O₃) presented a greater potential for 3D food printing, as demonstrated in Figure 6.

Moreover, starch with capacity to form strong gels shows potential to be use as a powerful gelling agent for food applications such as jams, jellies, puddings, and flans (OTEGBAYO; OGUNIYAN; AKINWUMI, 2014; IRANI et al., 2019). Additionally, the use of starches that form strong gels can be extent to the pharmaceutical and medical fields. Some examples are the production of capsules, tablets, and as an additive for the manufacturing of bone tissue engineering scaffolds with improved mechanical strength (FAURE; YORK; ROWE, 2001; KOSKI; BOSE, 2019). It is worth mention cassava starch is known as a source that produces weak gels, which limit their application – which, therefore, reinforce the relevance of the present results.

In contrast, the O_3 +DHT starch exhibited different behavior from the native and other modified starches, presenting a poor gel formation (Figure 6). Moreover, the O_3 +DHT hydrogel presented a "liquid" form. Starches with capacity to form weak gels are best thickeners for applications such as canned soups and filling for chocolate

confections (RAPAILLE; VANHEMELRIJCK, 1997; XIE; LIU; CUI, 2005). Moreover, starch gels that presents an elastic texture are undesirable for spray-drying since it can encumber the spraying process (IIDA et al., 2008), hence, the O₃+DHT starch could be used for this type of application. Also, weak gels has great potential in food application as basis of formulations where the target population has specific requirements, such as puree-based baby or elderly people based food (TORRES; CHENLO; MOREIRA, 2018).

Before discussing what influenced the texture results of the hydrogels based on the modified starches, first is necessary to understand the process of gel formation, which is mainly related to the retrogradation phenomenon.

Retrogradation is a process that occurs after cooling the hot gelatinized starch paste, which favors the formation of chain entanglements and the rearrangement of the starch molecules: initially, there is the formation of an amylose network during pate cooling and then it is observed the formation of ordered or crystalline amylopectin molecules during storage (BILIADERIS, 2009; WANG et al., 2015). Moreover, starch retrogradation is generally followed by other physical changes, for instance, increasing the consistency and turbidity of the pastes, gel formation, exudation of water, and increase of the degree of crystallinity (WANG et al., 2015).

Therefore, the process of gel formation is related to the starch retrogradation, whereby is observed a molecular re-association and the development of threedimensional network structures during storage at low temperatures (BILIADERIS, 2009). On the other side, modification technologies alter the molecular structure of starch: it can affect the molecular size distribution and introduces functional groups, which affects the starch retrogradation and gel formation. For instance, the presence of carbonyl and carboxyl groups influence the interactions of the molecules, by an attraction between carbonyl groups and repulsion between the carboxyl groups (CASTANHA et al., 2020). Furthermore, a better gel formation or a higher gel strength in modified starches, according to Handarini et al. (2020b), can be also related to improved interaction of amylose–amylose or amylose–amylopectin due to the additional hydrogen bonding between the functional groups. In this way, the gel formation of modified starches depends mainly on the size distribution of molecules, and the presence of functional groups.

Figure 8 proposes an explanation to correlate the hydrogels structure texture behavior, which is influenced by both the molecular size and functional groups.

Therefore, considering the molecular size distribution (Figure 4) and the carbonyl and carboxyl content, (Table 2) we hypothesized the following behavior: even though the O₃+DHT and DHT+O₃ starches presented similar molecular size distribution (Figures 4 – reduction of the first fraction), the hydrogel based on DHT+O₃ was strong, while the O₃+DHT one was weak. This behavior can be associated to the highest content of carbonyl groups of DHT+O₃ starch that contributed for better re-association of the starch molecules (amylose and amylopectin), once the carbonyl groups possibly result in hydrogen bridges with carbonyl or hydroxyl groups of starch chain segments, and these associations produce more structural integrity (HU et al., 2019). Moreover, for the inverse sequence (O₃+DHT), the molecular size distribution profile did not show a significant presence of intermediate-sized fraction, indicating that this modified starch is with a too-reduced molecular size which were not able to re-associate. Also, according to literature (CHAROENKUL et al., 2011; WANG et al., 2015), small-sized molecules favor the formation of strong gels, but if the molecules present a too-small size it can impair the molecular re-association, as well as the behavior observed for hydrogel based on O₃+DHT starch.





Figure 8. Schematic representation of the starch molecular size distribution and presence of functional groups in each treatment, as well as the effect on the hydrogels texture.

Finally, we observed that the dual combination can result in starches with differentiated properties (color and gel strength, in this case) in relation to modified starches obtained by single treatments or even the native starch. Moreover, we observed that the combined treatment DHT+O₃ exhibited a higher resistance to break than the other treatments, which can provide different industrial uses for this starch source. Also, if the aim is to obtain starches able to produce weaker gels, the inverse sequence of the combined treatments (O₃+DHT) showed this characteristic. In this way, the sequence of the treatments was very decisive for the performance of the hydrogel strength.

4. CONCLUSION

This work performed for the first time the combination of ozone (O_3) and dry heating treatment (DHT) for starch modification, investigating the changes in cassava starch structure and functionalities.

We observed that all modification processes did not modify the crystalline orthorhombic phase of the starch, but they caused changes in the amorphous domains. Furthermore, the dual treatment promoted different modifications in relation to the single treatments. Besides that, the sequence of these treatments was decisive for many properties of the modified starch obtained. The DHT+O₃ starch showed fissures on the granule surface, the greatest degree of oxidation, and a gel more resistant to break than the other hydrogels. On the other side, O₃+DHT starch showed a degree of oxidation similar to O₃ starch, molecular size distribution with very small-size molecules, and capacity to form weaker gels than the native. In general, ozone treatment was the process that more contributed to the starch oxidation performance (degree of oxidation and depolymerization). In fact, we obtained modified starches hydrogels that were ~6.6 fold stronger (based on the force to punctuate the gels) than the native starch gel, which expands the application potential of the cassava starch. Future works are necessary to explore possible applications of these modified starches and other combinations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Dâmaris Carvalho Lima: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. Bianca Chieregato Maniglia: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Data curation, Writing - review & editing. Manoel Divino Matta Junior: Formal analysis. Patricia Le-Bail: Resources, Project administration, Funding acquisition, Supervision, Writing - review & editing. Alain Le-Bail: Conceptualization, Resources, Project administration, Funding acquisition, Resources, Project administration, Funding acquisition, Writing - review & editing. Conceptualization, Resources, Project administration, Funding acquisition, Supervision, Writing - review & editing. Pedro Esteves Duarte Augusto: Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Funding acquisition, Project administration, Project administration, Project administration, Project administration, Project administration, Project administration, Project administration, Project administration, Project administration, Project administration, Project administraticon, Project administraticon, Project administratico

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Chapter 5: General conclusions and future studies
5.1 GENERAL CONCLUSIONS

This Thesis evaluated the ozonation process as a green technology to modify cassava and arracacha starches at different ozone concentrations and processing times. In addition, the combination of this technology with dry heating treatment was also evaluated to modify the cassava starch.

In general, it was observed that ozone is a highly oxidative technology once it was able to change the molecule size and functional groups, which affected the starch properties. It worth highlighting some of the desired changes in the starch properties; for instance, the modified cassava starch presented stronger hydrogels in special at acid conditions. Regarding the modified arracacha starch, it was observed it presented clearer pastes with stability over storage time. In this way, ozone technology is able to provide changes in the starch properties that can benefit its use in industrial products.

Furthermore, the combination of ozone (O₃) with dry heating treatments (DHT) in cassava starch provided distinct modifications concerning the single treatment (O₃ and DHT). Further, the sequence of these treatments had great influence in the starch properties: for instance, the hydrogel based on DHT+O₃ starch was more resistant to break than the other hydrogels, whereas the hydrogel based on O₃+DHT starch was weaker than the hydrogel based on native starch. Therefore, these starches presented interesting features that can be applied in the industry.

Finally, we highlight that, due all the results we observed in this Thesis, future studies are necessary to explore applications of these modified starches in different food and non-food products, evaluating their performance.

5.2 SUGGESTIONS FOR FUTURE RESEARCH

Even considering the promising results presented in this Thesis, it would be interesting further investigation of those technologies and the modified starches, in order to provide new insights and to favor their industrial use.

The ozone technology exhibited great potential to modify starches; however, as presented in Chapter 2, we applied ozone in excess in our system, which could be costly if used in the industry. Therefore, it highlights the importance of studying and understand the reaction, in order to optimize the system for the industry. Moreover, it would be interesting to evaluate this technology effects in other non-conventional sources, once we could demonstrate each source react differently.

Regarding the combination of technologies (DHT and O₃), the possibilities for future works is to further investigate in a molecular level, in order to understand why modification sequence influences differently the starch properties. Moreover, it would be interesting to evaluate the ozone processing combined with other technology, as also the optimization.

Finally, in relation to all the modified starches studied in this Thesis, it would be interesting to evaluate their application in other products, and their interaction with food additives and/or ingredients, as also with chemical compounds applied in other types of products, in order to promote these modified starches' application in different industry fields.

APPENDIX I: Simple Abstract (English, Portuguese, Spanish)

Simple abstract

Starch is a natural and versatile ingredient widely used in different industrial products. However, native starch presents some drawbacks that impair industrial use. Starch modification can be a good alternative to overcome these drawbacks. However, many technologies used to achieve it generate toxic wastes that can be harmful to the environment. Therefore, this Thesis studied two alternatives considered "environment-friendly" to modify starches: ozone (O₃) and its combination with dry heating (DHT). Each technology and process conditions resulted in a specific starch modification, with selected applications. For instance, the most relevant results are related with producing clearer and less viscous pastes, and stronger gels. Those properties can enhance the starch application in the industry, also adding value to specific sources.

Resumo simples

O amido é um ingrediente natural e versátil amplamente utilizado em diversos produtos industriais. No entanto, o amido nativo apresenta algumas desvantagens que prejudicam o uso industrial. A modificação do amido pode ser uma boa alternativa para superar essas desvantagens. No entanto, muitas tecnologias utilizadas para alcançá-lo geram resíduos tóxicos que podem ser prejudiciais ao meio ambiente. Portanto, esta Tese estudou duas alternativas consideradas de apelo ambiental para modificar amidos: o ozônio (O₃) e sua combinação com o aquecimento a seco (DHT). Cada tecnologia e condições de processo resultaram em uma modificação específica do amido, com aplicações selecionadas. Por exemplo, os resultados mais relevantes estão relacionados com a produção de pastas mais claras e menos viscosas e géis mais fortes. Essas propriedades podem potencializar a aplicação do amido na indústria, agregando também valor a fontes específicas.

Resumen simple

El almidón es un ingrediente natural y versátil muy utilizado en diferentes productos industriales. Sin embargo, el almidón nativo presenta algunos inconvenientes que perjudican el uso industrial. La modificación del almidón puede ser una buena alternativa para superar estos inconvenientes. Sin embargo, muchas tecnologías utilizadas para lograrlo generan desechos tóxicos que pueden resultar dañinos para el medio ambiente. Por ello, esta Tesis estudió dos alternativas consideradas de atractivo ambiental para modificar los almidones: el ozono (O₃) y su combinación con calentamiento en seco (DHT). Cada tecnología y condiciones de proceso dieron como resultado una modificación específica del almidón, con aplicaciones seleccionadas. Por ejemplo, los resultados más relevantes están relacionados con la producción de pastas más claras y menos viscosas y geles más fuertes. Esas propiedades pueden mejorar la aplicación del almidón en la industria, agregando también valor a fuentes específicas.

APPENDIX II: First page of the published articles



Ozone Processing of Cassava Starch

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ABSTRACT

This work evaluated the ozonation of cassava starch, correlating the process conditions with starch structure and properties. The starch structure was evaluated considering both molecules and granules. Ozonation resulted in an increased number of carbonyl and carboxyl groups with increasing ozone consumption. Moreover, a reduction of large and branched molecules was observed, with the consequent increase of smaller sized molecules. The structural changes significantly influenced the pasting properties, gel texture, water absorption and solubility indexes. As the main result, ozonation improved the hydrogels' texture. The 145.3 mg O_3 g_{starch}^{-1} sample produced gels ~280% stronger than the native starch. Moreover, the ozonated starch hydrogels maintained their consistency even at acidic conditions – on the contrary to native starch. Finally, the ozonated starch pastes were clearer than native ones. Therefore, ozone processing gives new possibilities for cassava starch application.

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Ozone; ozonation process; green technologies; starch modification; cassava starch

Introduction

Starches are natural ingredients relevant from an economic point of view since they are widely used in different industries, such as food (human and animal), pharmaceutic, cosmetic, chemical, petrochemical, paper, and textile, among others.

Starch granules, molecules, and trace elements may vary in form and functionality, according to its botanical origin. This variability provides starches with different physicochemical properties (Copeland et al. 2009). However, starches in their native form present limited uses for industrial application, such as poor shear and freeze-thaw stability, high degree of retrogradation, water insolubility, unstable pastes and gels (Ashogbon and Akintayo 2014; Zuhaib et al. 2018; Vanier et al. 2017; Zia-ud-Din and Fei 2017). Therefore, to overcome those limitations, different starch modification technologies have been used as an alternative to increasing their functionality for different applications.

Chemical, enzymatic and physical processes are used for starch modification. Chemical modification through acetylation, oxidation, cross-linking, hydroxypropylation and etherification are the most commonly applied (Vanier et al. 2017). However, by using chemical agents, different residues are produced, which can be unsafe for consumers, employees, and/or environment, or, at least, demand treatment and limit "clean labels". Ozone (O_3) is an attractive chemical technology for starch modification because it decomposes into oxygen, leaving no toxic residues in food or environment, and is considered a "green" and "environmentally friendly" technology with "clean label" (2019; Pandiselvam et al. 2017). Starch ozonation is further than a simple oxidation process, once this technology is able to promote important changes in both molecular size and chemical properties, leading to new properties and applications (Castanha, da Matta Junior, and Augusto 2017; Castanha et al. 2019).

Although starch modification using ozone was already studied for different sources, a relevant lack of knowledge is still observed.

For instance, some of the evaluated starches do not attend the industry's reality and demand, due to limited production, such as cocoyam, yam, lima bean, pigeon peas, jack bean and sago (Chan, Bhat, and Karim 2009; Chan et al. 2012, 2011; Oladebeye et al. 2013, 2018). Further, some starch sources have their use impaired, once they are associated with food allergies (Breuninger, Piyachomkwan, and Sriroth 2009; Sicherer 1999), such as corn and wheat starches (Çatal and İbanoğlu 2012a,

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Ozone modification of arracacha starch: Effect on structure and functional properties

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ABSTRACT

Arracacha starch presents interesting properties, such as a relatively low temperature of gelatinization and high paste clarity when compared with other commercial starches. Those characteristics can be very useful for industrial applications. However, the native arracacha starch presents some drawbacks that limit its further application, such as a weak gel and a high consistent paste. Therefore, modification techniques can expand its utilization. Ozone technology is a promising modification method, once studies reported that ozonation can decrease the apparent viscosity and increase the gel strength of different starch sources. Therefore, this work aimed to enhance arracacha starch properties (for instance, gel strength) by using ozone technology, in order to provide an alternative to the traditional oxidation chemical processes and to improve this starch's industrial applications. To achieve this, the effects of ozonation were evaluated in starch structure (granule and molecules) and properties, and possible industrial applications were suggested. The ozonation process promoted structural changes - reduction in the size of the molecules, formation of carbonyl and carboxyl groups, and an increase of reducing sugar content - that significantly affected the arracacha starch properties. Among the main results, it is worth highlighting the improved stability of paste clarity and hydrogels up to 250% stronger than the native one. Therefore, the ozonation process demonstrated to expand the arracacha starch industrial use.

1. Introduction

Arracacha (Arracacia xanthorrhiza Bancroft) is a root originally from the Andean South America region, and its production is mainly concentrated Colombia, Venezuela, Ecuador, Peru, Brazil and Bolivia (Albano, Franco, & Telis, 2014; Londoño-Restrepo, Rincón-Londoño, Contreras-Padilla, Millan-Malo, & Rodriguez-Garcia, 2018; Santos et al., 2018).

In general, the arracacha root is an "underutilized" crop (Blas, Hermann, & Baudoin, 2008; Leidi et al., 2018), presenting a low consumption and commercialization, even considering the production of bread, chips, soups, and for starch extraction (Londoño-Restrepo et al., 2018; Rocha, Cunha, Jane, & Franco, 2011). In fact, the isolation of starch from arracacha roots is an advantageous option to increase its commercialization, since the roots present a high content of starch, which is a great potential for industrial use (Castanha, Villar, Matta Junior, Anjos, & Augusto, 2018; Leonel et al., 2016; Londoño-Restrepo et al., 2018). In fact, Arracacha starch presents some interesting properties for specific industrial application, that will be mentioned below.

Arracacha starch has already been extracted and characterized by different researchers (Albano et al., 2014; Castanha et al., 2018; Leonel et al., 2016; Londoño-Restrepo et al., 2018). Castanha et al. (2018), for instance, evaluated starches from two varieties of Arracacha roots of the Andean Peruvian region and described some interesting characteristics for those starch samples, such as a high-water absorption and holding capacity, a low gelatinization temperature, and a high paste clarity compared with other commercial starches, i.e., cassava starch (Nwokocha, Aviara, Senan, & Williams, 2009). Those characteristics can be very useful for several industrial applications, being especially attractive for the food industry, since those properties are presented in a natural source - for instance, a starch with high paste clarity is excellent for using it in fruit pie fillings (Rapaille & Vanhemelrijck, 1997).

However, the native arracacha starch presents some drawbacks that limit its application in the industry, such as a weak gel and a paste with a

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Dual-process of starch modification: Combining ozone and dry heating treatments to modify cassava starch structure and functionality

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ABSTRACT

This work evaluated for the first time the effect of dual modification of cassava starch by using ozone (O_3) and dry heating treatment (DHT). The dual modification was capable to promote fissures on the surface of the starch granule (DHT + O_3), affected the starch amorphous domains, presented greater degree of starch oxidation (DHT + O_3) and different profiles of starch molecular size distribution. These modifications resulted in starches with different properties. Moreover, the sequence of treatments was decisive for the hydrogel properties: while DHT + O_3 resulted in formation of stronger gels, $O_3 + DHT$ resulted in weaker gels. In conclusion, this proposed dual modification was capable to produce specific modified starch when compared with the isolated treatments, also expanding the potential of cassava starch applications.

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1. Introduction

Starch is composed by two polysaccharides (amylose and amylopectin), as well as traces of proteins, fats, minerals, fibers, and water, organized in microparticles with both crystalline and amorphous regions called granules. In nature, starch is used as energy storage in plants, being found in different botanical sources (mainly cereals, pulses, roots, and tubers). From an economic point of view, starch is an important natural biodegradable ingredient for different industries, such as food, feed, cosmetic, chemical, pharmaceutical, paper, textile, petrochemical, among others. In food industry, starches are widely applied for thickening, texturizing, gelling, and stabilizing [1]. However, native starch has limited properties, which impairs its industrial application [2,3] - for instance, considering cassava starch, its weak gel can be pointed as an industrial limitation. Therefore, industries have been using different processes to modify and enhance starch functional properties.

The main starch modification technologies are: physical, chemical, enzymatic, and genetic. Chemical modifications (acetylation, oxidation, cross-linking, hydroxypropylation and etherification) are extensively used by the industry to modify native starches. However, it can be hazardous to the consumers and/or environment [4,5], or even limit applications (such as in food and pharmaceutical fields). Moreover, recently, consumer and industry preferences have focused on "environmentally friendly" modification technology and sustainable products [6].

Among these methods, ozone technology is considered an "environmentally friendly" chemical treatment for modifying starch, once ozone decomposes into oxygen without leaving toxic by-products [7–9].

Ozone as a starch modification technology has already been studied for different starch sources. Most of these studies observed that ozone processing has caused significant changes in the structure of starch as reduction of the size of amylose and amylopectin, and also replacement of the hydroxyl groups by carbonyl and carboxyl groups (through oxidation) [10]. In this way, ozone technology has been considered as a promising technology for starch modification once it has promoted improvements in starch functional properties. Considering cassava starch, ozone processing resulted in clearer pastes, stronger hydrogels even in acidic conditions [11], enhanced oven expansion and biscuits texture [12], enhanced biodegradable plastics [13], and 3D printed gels [14].

Another interesting "environmentally friendly" method is the dry heat/dry heating treatment (DHT), a physical modification method that does not use chemical reagents and only heat, thus being also considered the environment friendly [6,9].

In fact, this modification technology has been evaluated on different starch sources [4,15–23]. In the literature, it was observed that DHT can

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