UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ENGENHARIA DE ALIMENTOS

GRAZIELLE NÁTHIA NEVES

The use of clean technologies to obtain bioactive compounds from unripe genipap fruit (*Genipa americana* L.)

Uso de tecnologias limpas para a obtenção de compostos bioativos do jenipapo verde (*Genipa americana* L.)

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Thesis presented to the School of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Food Engineering.

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Engenharia de Alimentos.

Supervisor: Maria Angela Almeida Meireles Petenate

ESTE TRABALHO CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA GRAZIELLE NÁTHIA NEVES, E ORIENTADA PELA PROFA. DRA. MARIA ANGELA ALMEIDA MEIRELES PETENATE

CAMPINAS – SP
2019

Agência(s) de fomento e nº(s) de processo(s): CAPES, 001
ORCID: https://orcid.org/0000-0003-4561-7779

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Claudia Aparecida Romano - CRB 8/5816

Neves, Grazielle Náthia, 1988-
N414u The use of clean technologies to obtain bioactive compounds from unripe genipap (*Genipa americana* L.) / Grazielle Náthia Neves. – Campinas, SP : [s.n.], 2018.

Orientador: Maria Angela de Almeida Meireles Petenate.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. *Genipa americana* L.. 2. Corantes naturais. 3. Iridoides. 4. Tecnologias emergente. I. Petenate, Maria Angela de Almeida Meireles. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Uso de tecnologias limpas para a obtenção de compostos bioactivos do jenipapo verde *(Genipa americana* L.)
Palavras-chave em inglês:
Genipa americana L.
Natural colorants
Iridoids
Emerging technologies
Área de concentração: Engenharia de Alimentos
Titulação: Doutora em Engenharia de Alimentos
Banca examinadora:
Maria Angela de Almeida Meireles Petenate [Orientador]
Fabiana Queiroz
Julian Martínez
Irede Angela Lucini Dalmolin
Milena Martelli Tosi
Data de defesa: 15-03-2018
Programa de Pós-Graduação: Engenharia de Alimentos

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DEDICATION

To all my family, especially my Mother for never measuring efforts so that I could realize my dreams, for the love granted throughout my journey and for supporting all my decisions.

A toda minha família, em especial minha Mãe por nunca medir esforços para que eu pudesse realizar meus sonhos, pelo amor concedido ao longo de toda minha jornada e por apoiar todas as minhas decisões.

I dedicate this work...
Eu dedico este trabalho...

"Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas graças a Deus, não sou o que era antes".

Marthin Luther King

ACKNOWLEDGMENTS

This thesis becomes a reality with the kind support and help of many individuals. For this reason, I offer my sincere thanks:

Foremost, I want to thank GOD, who has always given me the strength, peace of my mind, good health and wisdom to face all the challenges.

I would like to express my sincere gratitude to my advisor Profa. M. Angela A. Meireles for the continuous support of my Ph.D. study and related research, for her patience, motivation, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D. study.

My sincere thanks also goes to Dra. Renata Vardanega, for her generosity and patience in sharing all knowledge with me. Without her precious support, it would not be possible to conduct this research. I'm lucky to have met you on my way. Thank you.

I would like to thank my friends Paulo and Suellen for being by my side during all the post-graduation.

I thank my fellow labmates, Gislaine, Eric, Pedro, Júlio, Abel, Tahmasb, Julia, Isabel, Monique, in for the stimulating discussions, for the days we were working together before deadlines, and for all the fun we have had in the last three years. Also I thank all my friends of the University of Campinas.

I would like to thank Ari for the daily help, patience and friendship.

I would like to thank the members of the examining committee, for the relevant contribution to the improvement of this work.

I would like to thank CAPES for the financial support.

Last but not the least, I would like to thank my family in particular my mom for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis.

In a way, I will take you all with me wherever I go, always with affection and gratitude.

RESUMO

Atualmente, o uso de corantes sintéticos predomina sobre os naturais em virtude do menor custo e sua maior estabilidade. Porém, o uso a longo prazo desses aditivos sintéticos pode acarretar sérios danos à saúde humana. Nesta perspectiva, os corantes naturais têm ganhado força e desafiado as indústrias a desenvolverem processos que garantam produtos naturais com estabilidade, viabilidade técnica e econômica, qualidade e segurança. O jenipapo verde (*Genipa americana* L.), um fruto nativo do Brasil, tem se destacado como uma boa fonte para a obtenção da um corante azul natural. Sendo assim, este trabalho propõe o uso do jenipapo verde para obtenção de um pigmento azul natural inexistente na indústria brasileira através de tecnologias de extração limpas e ambientalmente amigáveis. Isso porque este fruto é rico em genipina, uma substância que é capaz de produzir a cor azul. Com o intuito de quantificar a genipina e o geniposídeo, compostos responsáveis pala formação da cor azul no jenipapo, foi desenvolvido um método de cromatografia líquida simples e rápido que, com 13 minutos de corrida, permitiu separar estes compostos de forma eficiente em termos da resolução, seletividade e simetria dos picos. Em seguida, foi estudada a extração de diferentes partes do jenipapo verde utilizando etanol pressurizado. Os resultados obtidos neste estudo mostraram que a temperatura e as partes do fruto estudadas exercem grande influência na recuperação dos iridoides genipina e geniposídeo. A interação entre a pressão e as partes do fruto apenas influenciaram na recuperação do geniposídeo. O endocarpo e o fruto inteiro se destacaram como fontes para a obtenção de genipina, enquanto o mesocarpo e a casca se mostraram excelentes fontes para a recuperação de geniposídeo. Com o intuito de otimizar a extração do corante azul do jenipapo foi estudada a extração de genipina utilizando líquidos pressurizados (PLE), solvente a baixa pressão (LPSE) e prensagem a frio seguida por extração a baixa pressão (Press+LPSE). Neste estudo, os efeitos do solvente de extração (água e etanol), da temperatura (40, 50 e 60 °C) e da pressão (0,1, 2, 5 e 8 MPa) foram investigados, e apenas o solvente afetou significativamente a extração de genipina. O processo LPSE permitiu recuperar, em 25 minutos, 79 mg de genipina/g de jenipapo, enquanto o processo Press+LPSE recuperou neste mesmo tempo 83 mg de genipina/g de jenipapo. Além disso, o processo Press+LPSE recuperou mais de 90% do total de genipina em menos de 6 minutos. Ainda neste estudo, foi realizado uma avaliação econômica dos processos LPSE e Press+LPSE. Apesar de ambos os processos se mostrarem viáveis nos diferentes cenários analisados, o processo Press+LPSE apresentou maior viabilidade econômica com um tempo de retorno do investimento inferior a um

ano. Com o intuito de recuperar os compostos apolares do jenipapo verde, a extração utilizando dióxido de carbono supercrítico foi empregada. Neste estudo, os efeitos de temperatura (40 e 60 °C) e da pressão (15, 20, 25, 30 e 35 MPa) foram investigados, e apenas a pressão e a interação entre pressão e temperatura influenciaram no rendimento da extração. A melhor condição para a extração via SFE (extração com fluido supercrítico) foi 40 °C e 30 MPa. Os principais ácidos graxos encontrados neste extrato foram linoleico (13 ± 1 mg/g de jenipapo) e linolênico (2.2 ± 0.2 mg/g de jenipapo). Finalmente, o processo integrado foi estudado, no qual em um primeiro estágio foi obtido um extrato rico em ácidos graxos e num segundo estágio foi obtido um extrato rico em genipina (71± 6 mg/g de jenipapo). A etapa prévia de SFE não exerceu papel importante na LPSE, pois não possibilitou maior rendimento do composto genipina. Os processos desenvolvidos nesta tese mostraram-se tecnicamente eficientes na obtenção de um extrato que pode ser usado como um corante azul natural com propriedades funcionais pela indústria de alimentos, nutracêutica e de cosméticos.

Palavras-chave: *Genipa americana* L., corantes naturais, iridoides, tecnologias emergentes.

ABSTRACT

Currently, the use of synthetic colorants predominates over the natural ones due to the lower cost and its greater stability. However, the long-term use of these synthetic additives can cause serious damage to human health. In this perspective, natural colorants have gained strength and challenged industries to develop processes that guarantee natural products with stability, technical and economic viability, quality and safety. The unripe genipap (*Genipa americana* L.) a native Brazilian fruit has stood out as a good source for obtaining a natural blue colorant. Therefore, this work proposes the use of the unripe genipap to obtain a natural blue colorant that does not exist in the Brazilian industry through clean and environmentally friendly extraction technologies. That's because this fruit is rich in genipin a substance that is capable of producing blue color. In order to quantify the genipin and geniposide, compounds responsible for the formation of the blue color in genipap, a simple and fast liquid chromatography method was developed that with 13 minutes of running allowed to separate these compounds efficiently in terms of resolution, selectivity and symmetry of peaks. Then, the extraction of different parts of the green genipap was carried out using pressurized ethanol. The results obtained in this study showed that the temperature and the parts of the fruit studied exert a great influence on the recovery of the genipin and geniposide iridoids. The interaction between the pressure and the parts of the fruit only influenced the recovery of the geniposide. The endocarp and the whole fruit stood out as a source for obtaining genipin while the mesocarp and the peel proved to be excellent sources for the recovery of the geniposide. The extraction of genipin using pressurized liquids (PLE), solvent at low pressure (LPSE) and cold pressing followed by extraction at low pressure (Press+LPSE) was studied in order to optimize the extraction of blue colorant from genipap. In this study, the effects of extraction solvent (water and ethanol), temperature (40, 50 and 60 °C) and pressure

(0.1, 2.5, and 8 MPa) were investigated, and only the solvent significantly affected the extraction of genipin. The LPSE process allowed to recover 79 mg of genipin / g of genipap in 25 minutes while the Press+LPSE process recovered 83 mg of genipin / g of genipap at the same time. In addition, the Press + LPSE process recovered more than 90% of total genipin in less than 6 minutes. Also in this study, an economic evaluation of the LPSE and Press+LPSE processes was carried out. Although both processes prove viable in the different scenarios analyzed, the Press+LPSE process presented greater economic viability with a time of return of investment of less than one year. With the aim of recovering the apolar compounds from the unripe genipap, the extraction using supercritical carbon dioxide was used. In this study, the effects of temperature (40 and 60 °C) and pressure (15, 20, 25, 30 and 35 MPa) were investigated, and only the pressure and the interaction between pressure and temperature influenced the extraction yield. The best condition for SFE (supercritical fluid extraction) extraction was 40 °C and 30 MPa. The main fatty acids found in this extract were linoleic (13 ± 1 mg / g of genipap) and linolenic (2.2 ± 0.2 mg / g of genipap). Finally, the integrated process was studied, where in the first stage an extract rich in fatty acids was obtained and in a second stage an extract rich in genipin (71 ± 6 mg / g of genipap) was obtained. The previous SFE stage did not play an important role in the LPSE, since it did not allow higher yield of the genipin compound. The processes developed in this thesis have proved technically efficient in obtaining an extract that can be used as a natural blue colorant with functional properties by the food, nutraceutical and cosmetic industry.

Keywords: *Genipa americana* L., natural colorants, iridoids, emerging technologies.

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SUMMARY

- *CHAPTER 1* -

GENERAL INTRODUCTION, MOTIVATION, OBJECTIVES AND STRUCTURE OF THE THESIS

1.1 INTRODUCTION

Currently, industries of various segments have sought to change their product portfolio by replacing synthetic additives by natural ones to meet the demand of consumers who are more concerned about healthier eating habits. Colorants are present in products from food, chemical, pharmaceutical, textile industries, among others. Most of these industries predominantly use synthetic colorants due to their greater stability and lower cost of production when compared to natural colorants (Yamjala *et al.*, 2016). However, these colorants are said to cause epidermal, respiratory and cancerous diseases (Mirjalili *et al.*, 2011; Komissarchik and Nyanikova, 2014), which makes necessary to regulate and reduce their use.

In this sense, unripe genipap, a native fruit from Brazil that belongs to the *Rubiacea* family and to the *Genipa* genus arises as an alternative to obtain a natural blue colorant. Unripe genipap is rich in iridioids, among which genipin stands out for the ability to confer blue color. Genipin is a colorless iridoid that can be obtained directly from the genipap fruit with the use of organic solvents or after the enzymatic hydrolysis of the geniposide with β-glicosidases (Lee *et al.*, 2003; Ramos-De-La-Peña *et al.*, 2015). When the unripe genipap pulp is exposed to air it becomes gradually dark because when reacting with amino acids in the presence of oxygen the genipin turns blue (Lee *et al.*, 2003). This compound, found in the unripe fruits of genipap (*Genipa americana* L.), has been widely studied due to its pigment power of great industrial interest and also for its medicinal action that arouses the interest of pharmaceutical areas (Velásquez *et al.*, 2014).

It is worth mentioning that genipap is not only a source of iridoids, but also of phytosterols (Bailao *et al.*, 2015), phenolics (Souza *et al.*, 2012), anthocyanins (Souza *et al.*, 2012), flavonoids (Porto *et al.*, 2014), essential oils (Luzia, 2012), fatty acids (Figueiredo *et al.*, 1986) among others. These compounds present beneficial activities to the human body, and thus are of great industrial interest because it allows obtaining different products from a single raw material in an efficient and profitable way.

The use of green technologies to obtain natural products has been gaining ground in different industrial sectors because they allow obtaining promising products in an ecologically way. Green extraction consists of a process that reduces energy consumption, allows using safe solvents and renewable sources as raw material (Vazquez-Roig and Picó, 2015). The choice of the technology to be employed will depend on the characteristics of the plant matrix and the final product to be obtained.

Among the existing technological options, pressurized liquid extraction (PLE) is a green technique that appears as an alternative to obtain several compounds from solid or semisolid matrices (Subedi *et al.*, 2015). The first details of this technique were reported by Richter et al., (1996). Since then, it has gained space in the extraction scenario, being used and improved by several researchers. Extraction involving PLE uses liquid solvents at high pressures and temperatures, which makes the extraction process more efficient compared to traditional methods that use mild temperatures and ambient pressures (Mustafa and Turner, 2011). In addition to improving the extraction performance, PLE stands out for providing greater economic gain compared to traditional methods (soxhlet, maceration, percolation and sonication), since less amount of solvent and shorter processing time are required (Vazquez-Roig and Picó, 2015). It is a versatile technology that can be employed in a temperatures ranging from 313 to 473 K and in a pressure range between 3.5 - 35 MPa (Osorio-Tobón *et al.*, 2014). In addition to the temperature and pressure parameters, solvent selection is of fundamental importance for obtaining the compound of interest. Currently, water and ethanol are the most used solvents in PLE processes, as they are generally recognized as safe (GRAS) and allow the extraction of a range of polar compounds (Machado *et al.*, 2015).

However, in some time pressure does not have a positive effect to recover target compounds (Viganó *et al.*, 2016; Vardanega *et al.*, 2017). For this cases, the use of solvents at low pressures (ambient pressure) is suggested for the selective dissolution of target compounds contained in the solid matrix by a liquid solvent (Cardenas-Toro *et al.*, 2015). An advantage of this type of process over high pressure processes is its lower cost which makes it attractive for the food industry.

When the compound of interest is apolar, extraction using supercritical fluids (SFE) appears as an efficient green option for the recovery of bioactive compounds from several vegetable matrices (Viganó *et al.*, 2015). Currently, supercritical CO_2 is the most used fluid in SFE, since it has mild critical conditions (Tcritical = 31 °C and Pcritical = 7.38 MPa), it is available in large quantities, it is non-toxic, non-flammable, cheap, inert in various media and gaseous at atmospheric pressure (Da Silva *et al.*, 2016; Chemat *et al.*, 2017). The main parameters to be considered in SFE are temperature, pressure, particle size and moisture of the raw material, time of extraction, solvent flow rate of solvent and solvent-to-feed-ratio (Azmir *et al.*, 2013).

As mentioned before, one of the main factors that interfere in the choice for the process to obtain a particular product is its inherent costs, which means that in addition to being

efficient, the process also needs to be economically feasible. In this way, it is necessary to know all the details of the process to ensure that its industrial application is economically viable. The SuperPro Designer® Process Simulator (Intellingen, INC) is a software composed of models that represent industrial processes and allows the estimation of capital and operational costs in a given process (Carvalho *et al.*, 2015).

1.2 MOTIVATION

Nowadays, there is an industrial demand for developing more and more products with a high quality standard. Therefore, we must encourage our industries not only to develop potential products, but to implement this development in a sustainable way. In this context, the extraction of blue colorant from unripe genipap is a challenge for current science due to its low exploitation. There is a lack of naturally occurring blue-colored metabolites compared to other colors, and it is undeniable how useful a natural blue colorant would be for the food industry, which uses this coloring in candy, gum, and gelatine; products mainly aimed to children. The blue color is also present in the polymer, textile and cosmetics industries, and in the pharmaceutical industry that uses this coloration in various medicines. In adittion to genipin, the unripe genipap is a soruce of essential fatty acids such as linoleic and linolenic acids. The extraction of these fatty acids is very interesting because they cannot be synthesized by animals, including humans. Thus, this work proposed to develop a sustainable integrated extraction process that allows obtaining a genipin-rich extract and a fatty acid-rich extract. In addition, the economic analysis performed may encourage industries to use the extraction techniques addressed in this work.

1.3 OBJECTIVES

1.3.1 Main Objective

To accomplish a technical and economic evaluation of the process of obtaining natural blue colorant from unripe genipap fruit and to evaluate the composition of the recovered extracts.

1.3.2 Specific Objectives

- ✓ To obtain, identify and quantify the bioactive compounds present in the extracts from different parts of genipap using pressurized liquid extraction;

- ✓ To identify the best extraction conditions (temperature, pressure and solvent) based on genipin yield from genipap;
- ✓ To determine the kinetic parameters of low pressure extraction (LPSE) and cold pressing followed by LPSE (Press + LPSE) extraction of genipin;
- ✓ To verify the economic viability of the LPSE and Press + LPSE processes in terms of genipin yield;
- ✓ To optimize the extraction conditions (temperature and pressure) of the genipap extract using SFE;
- ✓ To integrate the SFE-LPSE processes in order to obtain a fatty acids rich-extract in a first stage and a genipin rich-extract in a second stage;

1.4 STRUCTURE OF THE THESIS

The development stages of the research project are presented in 8 chapters. In this **Chapter 1 - Introduction, Motivation, Objectives and Structure of the Thesis -** the main subject of the study, the intended objectives and the steps involved in its accomplishment are presented. The activities performed are presented in Figure 1.

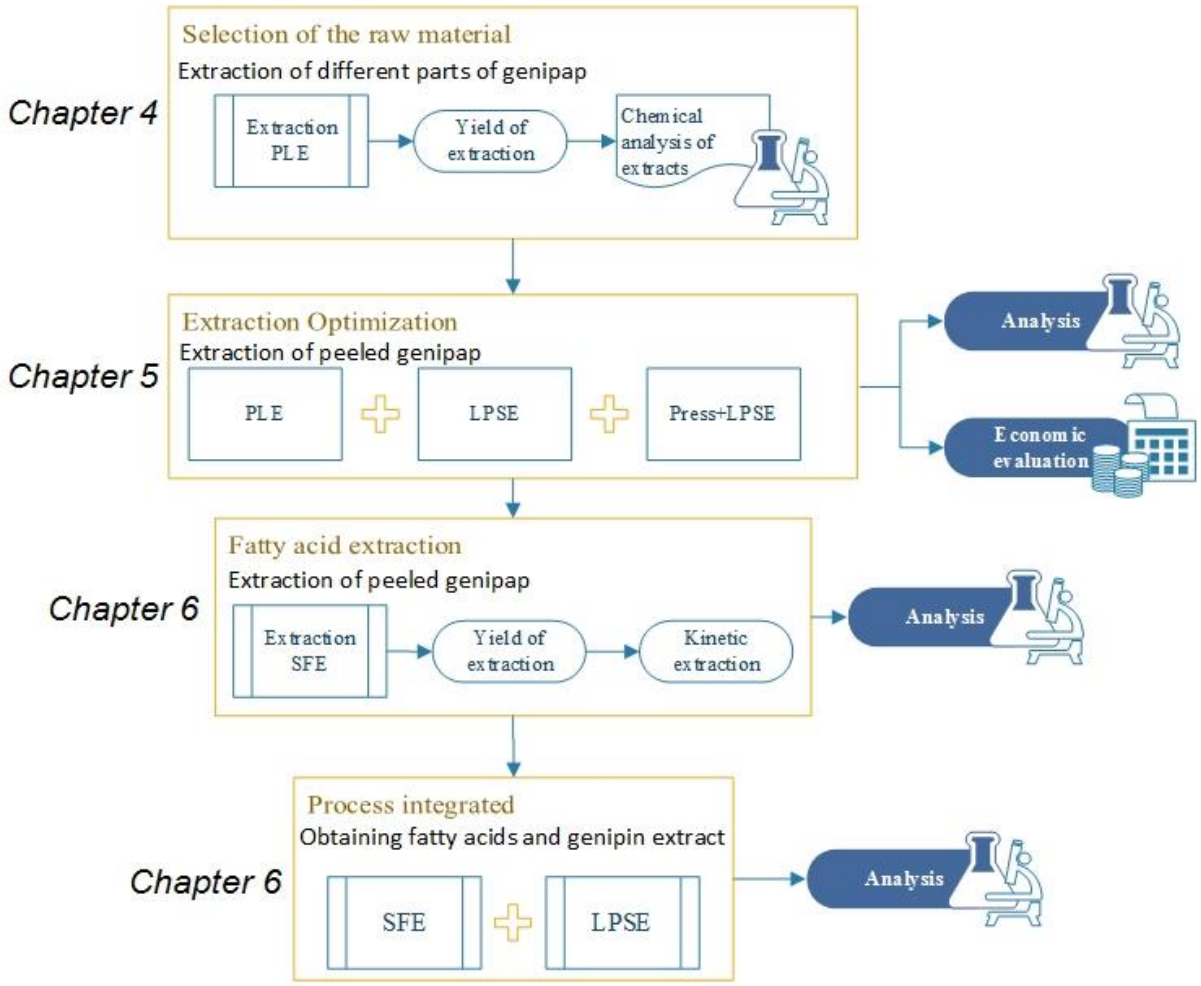

Figure 1 - Flow chart of the activities carried out in this thesis.

In **Chapter 2 - Genipap: a new perspective on natural colorants for the food industry -** is a review on the use of genipap as source for obtaining the natural blue colorant is presented. In this paper, the physico-chemical characteristics of genipap and the iridoids of this fruit are presented. Techniques used for the extraction of bioactive compounds are also mentioned, as well as details of the history of works that discuss the obtaining of genipin from genipap fruit. Finally, the benefits of genipin to human health and some suggestions for future research are presented.

Chapter 3 - Identification and quantification of genipin and geniposide from Genipa americana L. by HPLC-DAD using a fused-core column – presents the details of the methodology of analysis of geniposide and genipin by high performance liquid chromatography (HPLC). The methods reported in the literature for quantification of these compounds present long run times. To overcome this drawback, a robust analytical method was developed and validated to quantify genipin and geniposide, with a total analysis time of 13

min. This method was used to identify and quantify genipin and geniposide in the studies presented in chapters 4, 5 and 6.

In **Chapter 4 - Extraction of bioactive compounds from genipap (*Genipa americana* L.) by pressurized ethanol: Iridoids, phenolic content and antioxidant activity -** the experimental results of the study that evaluated the extraction of bioactive compounds from different parts of unripe genipap using pressurized ethanol are presented. The studied parts of genipap were the whole fruit, the pell, the mesocarp, the endocarp, the endocarp + seeds and the seeds, and the effects of temperature (50 and 80 °C) and pressure (0.2, 1.2 and 2.0 MPa) genipap on global yield and the recovery of genipin from the different parts of genipap were investigated. The results obtained in this study were extremely important for the accomplishment of the next steps, because although the endocarp is known as the part with the highest content of genipin, this part represents only 12% of the whole fruit, which could make it unfeasible on an industrial scale.

In order to increase the genipin yield, in **Chapter 5 - Extraction of natural blue colorant from *Genipa americana* L. using green technologies: Techno-economic evaluation -** the extraction using the whole fruit without the peel was performed, because it was observed in preliminary tests that the removal of the genipap peel favor the obtaining of an extract of blue coloration without compromising the content of genipin. Therefore, in this chapter the experimental results for the extraction of genipin from the whole fruit of genipap (without peel) are presented. The variables studied were pressure (0.1, 2, 5 and 8 MPa), temperature (40, 50 and 60 °C) and solvent (water and ethanol). The kinetic behavior of two processes (LPSE and Press + LPSE) was evaluated and an economic study was performed in order to verify the economic feasibility of genipin extraction in different scenarios.

In addition to genipin, the unripe genipap fruit is also a source of non-polar compounds such as fatty acids. Therefore, in **Chapter 6 - Obtaining fatty acids and genipin from *Genipa americana* L. in a biorefinery concept: SFE process integrated with low-pressure solvent extraction -** the extraction using supercritical CO_2 (SFE) to obtain fatty acids of unripe genipap was investigated. The effects of temperature (40 °C and 60 °C) and pressure (15, 20, 25, 30 and 35 MPa) were evaluated. In order to make the most of the biomass, the integration of the SFE and LPSE processes was studied, where in the first stage an extract rich in fatty acids was obtained and the biomass resulting from this process was subjected to extraction with liquids at low pressure to obtain a genipin-rich extract.

The **Capítulo 7** – **General discussion -** brings an integrated discussion of all the chapters previoulsy presented and the most relevant results obtained in chapters 3, 4, 5 and 6 thus improving the general understanding of the thesis. **Chapter 8 - General conclusions and suggestions for future research -** presents the conclusions that could be obtained during the development of the thesis as well as presents some suggestions for future research.

In **the Memory of the period of doctorate** are listed scientific papers published in periodicals and in annals of events resulting from the project and co-authorship, as well as the courses and stages of teaching. The APPENDIX contains non publicated materials.

REFERENCES

AZMIR, J. et al. Techniques for extraction of bioactive compounds from plant materials: A review. **Journal of Food Engineering,** v. 117, n. 4, p. 426-436, 2013. ISSN 02608774.

BAILAO, E. F. et al. Bioactive Compounds Found in Brazilian Cerrado Fruits. **Int J Mol Sci,** v. 16, n. 10, p. 23760-83, 2015. ISSN 1422-0067 (Electronic)
1422-0067 (Linking).

CARDENAS-TORO, F. P. et al. Pressurized liquid extraction and low-pressure solvent extraction of carotenoids from pressed palm fiber: Experimental and economical evaluation. **Food and Bioproducts Processing,** v. 94, p. 90-100, 2015. ISSN 09603085.

CARVALHO, P. I. N. et al. Techno-economic evaluation of the extraction of turmeric (Curcuma longa L.) oil and ar-turmerone using supercritical carbon dioxide. **The Journal of Supercritical Fluids,** v. 105, p. 44-54, 2015. ISSN 08968446.

CHEMAT, F. et al. Review of Green Food Processing techniques. Preservation, transformation, and extraction. **Innovative Food Science & Emerging Technologies,** v. 41, p. 357-377, 2017. ISSN 14668564.

DA SILVA, R. P. F. F.; ROCHA-SANTOS, T. A. P.; DUARTE, A. C. Supercritical fluid extraction of bioactive compounds. **TrAC Trends in Analytical Chemistry,** v. 76, p. 40-51, 2016. ISSN 01659936.

FIGUEIREDO, R. W. et al. Características físicas e químicas do jenipapo. **Pesquisa Agropecuária Brasileira,** v. 21, n. 4, 1986.

KOMISSARCHIK, S.; NYANIKOVA, G. Test systems and a method for express detection of synthetic food dyes in drinks. **LWT - Food Science and Technology,** v. 58, n. 2, p. 315-320, 2014. ISSN 00236438.

LEE, S.-W. et al. Colorimetric determination of amino acids using genipin from Gardenia jasminoides. **Analytica Chimica Acta,** v. 480, n. 2, p. 267-274, 3/24/ 2003. ISSN 0003-2670.

LUZIA, D. M. M. Propriedades funcionais de óleos extraídos de sementes de frutos do cerrado brasileiro. 2012.

MACHADO, A. P. D. F. et al. Pressurized liquid extraction of bioactive compounds from blackberry (Rubus fruticosus L.) residues: a comparison with conventional methods. **Food Research International,** v. 77, p. 675-683, 2015. ISSN 09639969.

MIRJALILI, M.; NAZARPOOR, K.; KARIMI, L. Eco-friendly dyeing of wool using natural dye from weld as co-partner with synthetic dye. **Journal of Cleaner Production,** v. 19, n. 9-10, p. 1045-1051, 2011. ISSN 09596526.

MUSTAFA, A.; TURNER, C. Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. **Anal Chim Acta,** v. 703, n. 1, p. 8-18, Oct 3 2011. ISSN 1873-4324 (Electronic) 0003-2670 (Linking).

OSORIO-TOBÓN, J. F. et al. Extraction of curcuminoids from deflavored turmeric (Curcuma longa L.) using pressurized liquids: Process integration and economic evaluation. **The Journal of Supercritical Fluids,** v. 95, p. 167-174, 2014. ISSN 08968446.

PORTO, R. G. C. L. et al. Chemical Composition and Antioxidant Activity of Genipa Americana L. (Jenipapo) of the Brazilian Cerrado. **Journal of Agriculture and Environmental Sciences,** v. 3, n. 4, 2014. ISSN 23342404
23342412.

RAMOS-DE-LA-PEÑA, A. M. et al. Recovery of genipin from genipap fruit by high pressure processing. **LWT - Food Science and Technology,** v. 63, n. 2, p. 1347-1350, 2015. ISSN 00236438.

RICHTER, B. E. et al. Accelerated Solvent Extraction: A Technique for Sample Preparation. **Analytical Chemistry,** v. 68, n. 6, p. 1033-1039, 1996/01/01 1996. ISSN 0003-2700.

SOUZA, V. R. et al. Determination of bioactive compounds, antioxidant activity and chemical composition of Cerrado Brazilian fruits. **Food Chemistry,** v. 134, n. 1, p. 381-386, 2012. ISSN 03088146.

SUBEDI, B. et al. Selective pressurized liquid extraction as a sample-preparation technique for persistent organic pollutants and contaminants of emerging concern. **TrAC Trends in Analytical Chemistry,** v. 68, p. 119-132, 2015. ISSN 01659936.

VARDANEGA, R.; SANTOS, D. T.; MEIRELES, M. A. A. Proposal for fractionating Brazilian ginseng extracts: Process intensification approach. **Journal of Food Engineering,** v. 196, p. 73-80, 2017. ISSN 02608774.

VAZQUEZ-ROIG, P.; PICÓ, Y. Pressurized liquid extraction of organic contaminants in environmental and food samples. **TrAC Trends in Analytical Chemistry,** v. 71, p. 55-64, 2015. ISSN 01659936.

VELÁSQUEZ, C. L.; RIVAS, A.; OCANTO, I. S. Obtención de Genipina a partir de frutos de caruto (Genipa americana L.) del llano venezolano. **Avances en Química,** v. 9, n. 2, p. 75-86, 2014. ISSN 1856-5301.

VIGANÓ, J. et al. Pressurized liquids extraction as an alternative process to readily obtain bioactive compounds from passion fruit rinds. **Food and Bioproducts Processing,** v. 100, p. 382-390, 2016. ISSN 09603085.

VIGANÓ, J.; MACHADO, A. P. D. F.; MARTÍNEZ, J. Sub- and supercritical fluid technology applied to food waste processing. **The Journal of Supercritical Fluids,** v. 96, p. 272-286, 2015. ISSN 08968446.

YAMJALA, K.; NAINAR, M. S.; RAMISETTI, N. R. Methods for the analysis of azo dyes employed in food industry - A review. **Food Chem,** v. 192, p. 813-24, Feb 1 2016. ISSN 0308-8146 (Print) 0308-8146 (Linking).

- *CHAPTER 2* -

LITERATURE REVIEW

Genipap: a new perspective on natural colorants for the food industry

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Article published in the journal Food and Public Health, vol. 8., p. 21-33, 2018

ISSN: 2162-9412. DOI: 10.5923/j.fph.20180801.04

Article available in: http://article.sapub.org/10.5923.j.fph.20180801.04.html

Food and Public Health 2018, 8(1): 21-33
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Genipap: A New Perspective on Natural Colorants for the Food Industry

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Abstract The colors in food attract the attention of consumers, trigger emotions and generate expectations about food. Currently, the use of synthetic colorants is more common than natural ones due to their lower cost and greater stability. However, the long-term use of these synthetic additives can cause serious damage to human health. Currently, no colorant with a natural source is used at an industrial scale for obtaining blue pigments. Therefore, it is highly important to find a new source of blue color because food industries use it in many products, such as ice cream, chocolate and candies, which are mainly products intended for children. This review focused on the use of genipap as an alternative for obtaining a natural blue pigment for use in food industries. Additionally, techniques are described for extraction, and the stability of blue pigments and health properties of genipin are discussed. At the end of the review, it was observed that a stable blue pigment can be obtained from genipap. In addition to coloring, these pigments have medicinal properties of great interest to the pharmaceutical industry.

Keywords *Genipa americana* L., Genipin, Color additives, Blue pigments

1. Introduction

Currently, natural products with functional properties have attracted the interest of many industries because synthetic additives are increasingly being replaced with natural additives to attract consumers who have healthy eating habits. Colorants are additives that are present in almost all food products. There is demand from regulatory agencies to reduce the use of synthetic colorants, because they may be responsible for respiratory, epidermal and carcinogenic diseases [1, 2]. In this context, there is an industrial interest in natural colorants, which have limited use in industry due to their instability when exposed to light, pH changes and oxygen [3].

Color additives can be classified according to their origin (natural or synthetic), covering (opaque or transparent) and their solubility (dyes or pigments) [4]. Although dyes are soluble in the medium in which they are applied, pigments are insoluble in common solvents [4, 5]. According definition by FDA (Food & Drug Administration) a color additive is any dye, pigment, or other substance that can impart color to a food, drug, or cosmetic or to the human body. Thus, color additives are classified as straight colors that have not been mixed or chemically reacted with any other substance; lakes that are formed by chemically reacting straight colors with precipitants and substrata; and mixtures that are formed by mixing one color additive with one or more other color additives or non-colored diluents, without a chemical reaction. In addition, any chemical that reacts with another substance and causes formation of a color may be a color additive [6].

Several vegetable matrices are used to obtain a range of colorants. Annatto (*Bixa orellana*), for example, is used to extract colors ranging from yellow to red [7]. The extracts from jabuticaba (*Myrciaria cauliflora*) contain anthocyanins, which are phenolic compounds that generate blue, purple and red colors [8]. The yellow-orange color can be obtained from curcuminoids present in the species *Curcuma Longa* [9]. However, there is difficulty in obtaining a stable blue color from raw vegetable materials.

From this perspective, the genipap (*Genipa americana* L.), which is a native fruit from Brazil, is an alternative for obtaining a natural blue pigment [10]. The blue pigments from unripe fruits of genipap have been shown to highly stable and have promising applications in food and non-food products [11]. The food industry, for example, utilizes blue coloring in several products and to obtain other colors, such as purple and violet [12].

One of the factors that limit the use of natural colorants is their stability. In general, natural additives are less stable than synthetic ones. This instability has encouraged researchers from around the world to search for new technologies applicable to the food and beverage market to obtain non-toxic colorants that are safe to use in food [13, 14].

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Published online at http://journal.sapub.org/fph

Thus, this review aims to cover the general aspects of using genipap as a new source for obtaining blue colorants in the food industry. Furthermore, this review includes a brief description of the techniques used for genipin extraction and discusses the challenges faced by the industry in using natural colorants as well as future possibilities for using genipap-based colorants in food products.

2. Colorants in Food

Color is one of the attributes that is most valued by consumers when purchasing food. To ensure food has an attractive and durable appearance, coloring agents are added to food.

Regardless of origin, whether they are natural or synthetic, color additives must:

i) Comply with the requirements imposed by regulatory agencies. In Brazil, there are laws that must be followed for the addition of natural and artificial colorants, and a correct description of these additives must be included on the label of food products, such as Decree n° 55871 of March 26th, 1965 [15]; Decree n° 50040, January 24th, 1961 [16]; Resolution n° 37/77 [17]; Resolution n° 44/77 [18]; RDC n° 259/2002 [19]; and Resolution n° 340/2002 [20]. The National Health Surveillance Agency (ANVISA) is the Brazilian organization that regulates the application of 41 food colorants, of which 21 are natural and 20 are synthetic [21], and both types must be within the concentration limits that are necessary for consumer safety [17]. In Europe Union (EU) the regulation (EC) No. 1129/2011 include the rules for food colors; the annexes of the Regulation (EC) No. 1333/2008 contain food categories and a positive list of colors permitted, quantities and instructions for use. Natural pigments should be used in accordance with the rules of the Regulation (EC) No. 178/2002 and other applicable rules [22]. The EU, through Directive 95/45/EC, 1995, authorized the use of 43 colorants in food applications, which includes 17 synthetic and 26 natural colorants [23]. In the United States of America (USA) the rules for food colorants are available under the Title 21 of the Code of Federal Regulations (21 CFR), which contain rules on petitions and labelling and list the specifications and rules for use of approved color additive [22]. The list of colorants permitted in USA is divided into two categories: (*i*) color additives certified by the FDA, which include 9 additives and (*ii*) color additives exempt from certification by the FDA, which includes 27 additives for a total of 36 additives permitted by FDA [24].

ii) Be stable to prevent degradation of the colorant throughout distribution and sale. The main causes of colorant instability include heat, light, oxygen, acid and exposure to oxidizing agents, such as ascorbic acid and trace metals [3].

2.1. Synthetic Colorants

Synthetic colorants are produced by complete chemical synthesis or by chemical modification of various precursor compounds [25]. These colorants are widely used in food production because they improve the visual and sensory characteristics of food as well as promoting their marketing.

Although they have greater stability, lower production cost and are easier to manage than natural ones, the use of these additives can cause toxic effects at short and long terms for human, e.g. by promoting hyperactivity in children and by their possible carcinogenic effects [11, 27]. Furthermore, the synthetic colorants have been blamed to be harmful to the environment because when they are not being fixed in the food matrix, these colorants pass to the industrial effluent, which when released into water bodies represent a threat to the environment [28].

Among the synthetic colorants used in the food industry, azo colorants account for 65%. These colorants are characterized by the presence of nitrogen and provide vivid and intense colors that make their use very common in food, textile, leather and cosmetics [29]. It is estimated that over 10,000 different dyes and pigments are used industrially, and over 7 × 105 tons of synthetic dyes are annually produced worldwide [30].

2.2. Natural Colorants

The natural colorants obtained from plants, insects, and minerals are characterized by being renewable and sustainable products [31]. The ability to make natural colorants is a technique that has been used since ancient times and has been investigated in recent years due to concerns about the environment and human health [1, 32]. Natural colorants are biodegradable, non-toxic and non-carcinogenic [1].

There have been many advances in developing natural food colorings with respect to extraction processes, purification, stability, identification of new sources, formulation techniques, and hygiene and safety criteria. Nonetheless, there is still a need for developing new natural colorants with high stability and good coloring strength that have wide industrial applications [31].

Currently, several natural colorants are obtained from vegetable matrices. There are many natural colorants applied in commercial foods, these colorants include carotenoids, anthocyanins, chlorophyll and betalains that in addition to providing pigments, perform functional activities in the human body. The chemical structures of some plant-based pigments are shown in Figure 1 [33].

Carotenoids are natural pigments metabolized by plants, algae and photosynthetic bacteria responsible for yellow, orange and red tones in some fruits and vegetables [34]. These pigments are soluble in lipid compounds and perform important roles in human health by preventing

cardiovascular diseases and protecting against some cancers [35, 36]. Carotenoids like β-carotene, lutein, violaxanthin, neoxanthin, β-cryptoxanthin, fucoxanthin, lycopene and astaxanthin extracted from plants, algae and even insects have been employed in food products such as sauces, marinades, spice blends, coatings, beverages and milk [37].

Among vegetable matrices rich in carotenoids, there is annatto, which is a native plant from the tropics of South America that belongs to the family *Bixa Orellana* [38]. The seeds of this plant provide a very important source of pigment for the food industry because the seeds are rich in two carotenoids: bixin (80%) and norbixin (20%), which provide colors that range from yellow to red [7, 39]. Annatto is a permitted natural food colorant (E number E160b) and their carotenoids bixin and norbixin are used in food products such as cakes biscuits, rice, dairy products, flour, fish, soft drinks, snacks and meat products [37].

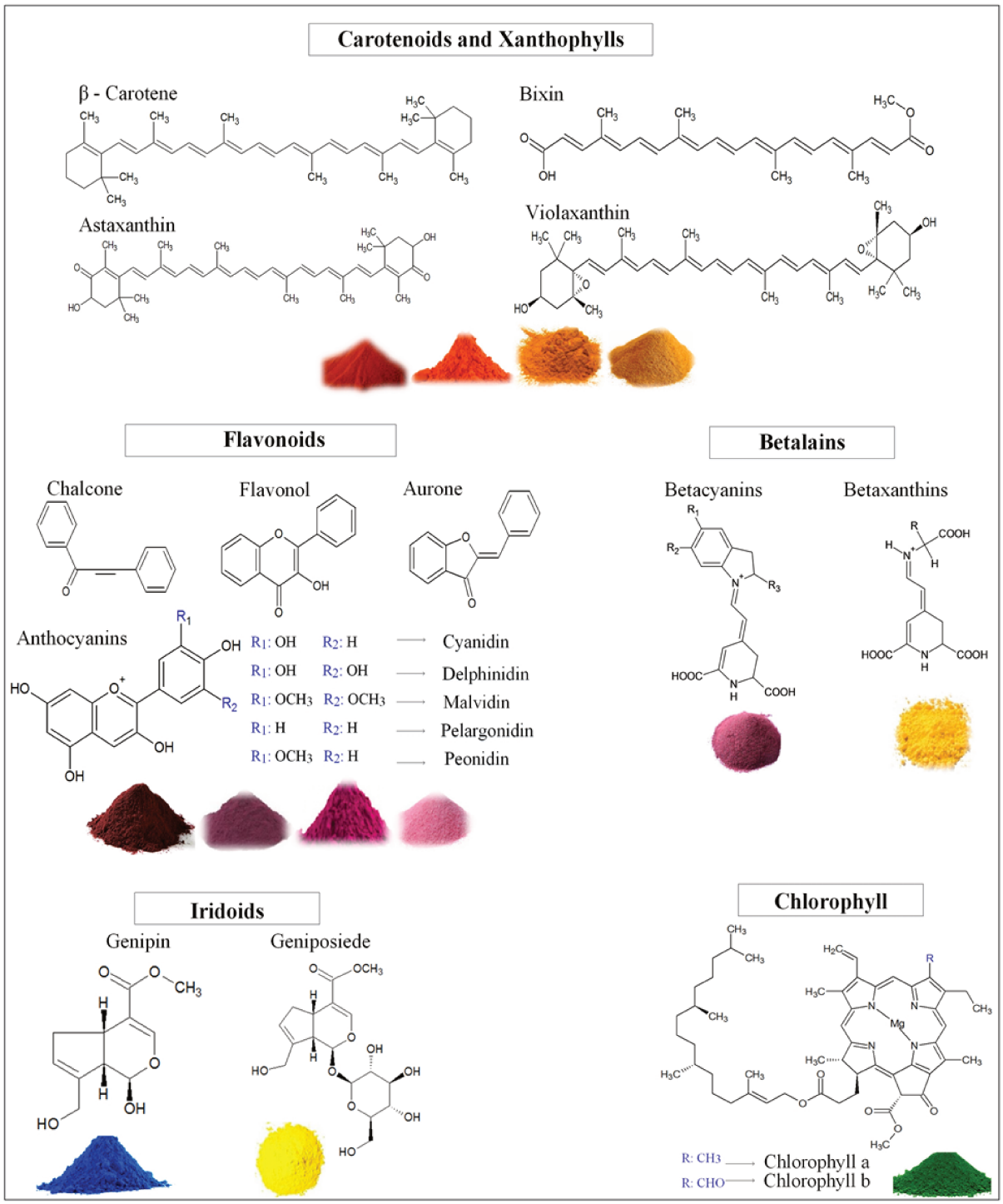


Figure 1. Natural pigments from different vegetable matrices

Anthocyanins are one of the most important groups of pigments found in nature. These compounds belong to the flavonoids group and are responsible for the color of a wide variety of fruits, leaves, and flowers. In addition to coloring, anthocyanins have antioxidant, anti-inflammatory and anticancer properties [40].

The anthocyanins present in some berry fruits, such as red cabbage and purple sweet potato provide pigments in purple, violet, and blue hues [26]. The main anthocyanins found in nature are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin. Anthocyanins (E 163) have been used as colorants in soft drinks, confectionary products and fruit preparations [37]. The use of anthocyanins as a food coloring has been limited due to their low stability and interactions with other compounds present in foods [26].

Chlorophylls are vegetable pigments that occur naturally in plants and confer a green color [37]. Due to its complex structure, difficult stabilization, susceptibility to photobleaching and low stability in an acidic medium (pH = 3.5-5.0), the use of chlorophylls as a food coloring has been limited. Among the types of chlorophyll (a, b, c_1, c_2 and d), only the types a and b are used as industrial colorants [26, 37]. Chlorophylls (E 140) has application in dairy products, soups, drinks and sugar confections [37].

Unlike carotenoids, anthocyanins and chlorophyll, betalains have received less attention from researchers [41]. Betalains are plant pigments that belong to the order Caryophyllales and are often studied as anthocyanins. According to their chemical structure, betalains can be divided into betacyanins (red-violet color) and betaxanthins (yellow color) [26, 42]. The main source of betaine is beetroot (*Beta vulgaris*), which is also the only source of betalains allowed for food production. Betalains have greater applicability in the food industry than anthocyanins because they produce three times more intensity than anthocyanins [37, 42]. Additionally, betalains are stable at a higher pH range between 3 and 7 [43]. Betalain derived from beetroot (E 162-betanin) are used in dairy products and meat products [37].

Recently, phycocyanin (which is a blue-green pigment extracted from algae) in the form of a *Spirulina platensis* extract was approved for coloring sweets and gums. However, its use is limited by its instability when exposed to heat, light and acidic mediums [3]. Blue pigments are also produced by iridoid derivatives from genipap and *Gardenia jasminoides*. These colorants are more stable when exposed to heat, light, and pH changes compared to pigments obtained from phycocyanin [44].

3. Challenges Facing the Food Industry in Obtaining Natural Colorants

Over the last few decades, a progressive evolution of the food industry has been observed regarding the development of products with natural additives [27]. Motivated by the growing demands of modern consumers, the industrial sector has been increasingly required to offer naturally colored products that not only generate sensory interest but also have potential benefits for human health [26]. The three major challenges industries for obtaining natural pigments are described below [26, 31, 37, 45]:

- ✓ Stability: Usually synthetic colorants are more stable than natural ones for a larger range of pH values, temperature variations as well as exposure light and oxygen;
- ✓ Efficiency: The efficiency of natural additives is also crucially important for the industrial sector since the amounts of additives must be calculated so that the additive performs its role without decreases in product quality and consumer welfare. Often, higher quantities of natural additives are required compared to synthetic additives and it may not be cost effective or advisable from a health security point of view. Another limitation is the range of tones that are available naturally;
- ✓ Cost: The high cost of obtaining natural compounds is another factor that limits the manufacture of products using natural colorants. The cost for recovery and purification of a particular natural compound is often much higher and this cost will be transferred to the final product, which makes it less competitive in the marketplace.

Currently, there is no natural colorants production enough to supply the demand of the food industry for natural colorants. Thus, to reach the full production demand needed it is mandatory to invest in research and development in order to find abundant sources of natural colorants which make its application technical and economically feasible.

However, it is not enough to develop only a product with appealing color, flavor, appearance, texture and odor attributes. It is necessary that the product provide security for the consumer and not cause harm to their health after ingestion. Therefore, it is necessary for food manufacturers to comply with existing laws. These laws are regularized by different agencies according to each country, e.g., FDA (USA), EFSA (European Union), ANVISA (Brazil).

These different laws from each country often represent a barrier to industry since they limit, for example, the marketing of products between different countries.

For many years, the use of genipin as a colorant was limited to only a few Asian countries, such as Japan and Korea. More recently, the genipin colorant has been reported as a "fruit juice" color additive in the United States (Title 21 CFR, Code of Federal Regulations, § 73.250) [46] and was approved for food in Colombia [11].

4. Genipap as Source of Blue Pigments

Genipap is a fruit belonging to *Rubiaceae* family, which is widely distributed throughout Central America and South America [47]. The fruit has different names according to its place of origin, i.e., in Spanish-speaking regions, it is known

as *jagua*, *juito*, *huito*, *genipa* or *caruto*, in English-speaking regions, the terms genipap or genipa are names for this fruit, and in Portuguese-speaking regions, such as Brazil, it is popularly known as *jenipapo*.

The genipap tree is an evergreen tree with a height of approximately 10-20 m. The fruits are comestible and globular with a diameter of 5-8 cm and weights ranging from 200-400 g [48, 49]. When in its ripe stage, the pulp is succulent, acidic and hard. The outside of the fruit has a gray-yellowish, dark brown or greenish color [48, 50]. Its pulp is aromatic and mushy.

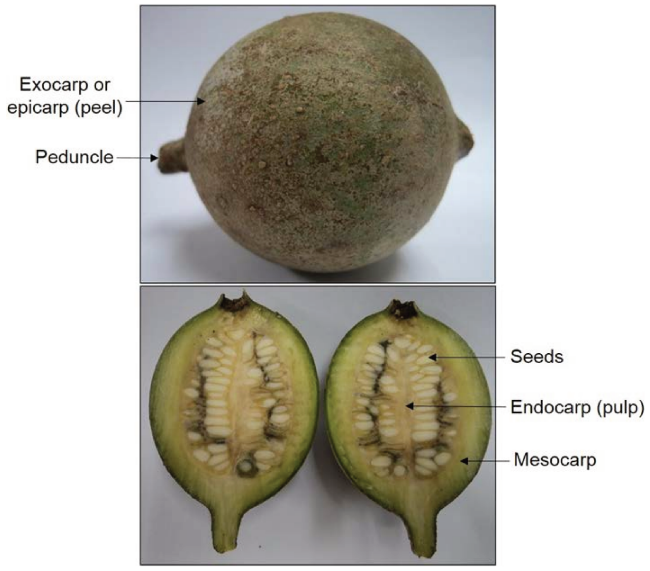


Figure 2. Morphology of the unripe genipap fruit

This fruit has numerous albuminous seeds that are hard and have a fibrous consistency with a dark brown color and length ranging from 6 to 12 mm, and the seeds are protected within the fruit by fresh pulp [50]. With an unusual aroma and taste, this fruit is popularly consumed in juices, jams and liqueurs. On the other hand, when in its unripe stage, these fruits may be used as a source of tissue colorants, body paints and food colorings [51]. Figure 2 illustrates the morphological characteristics of the genipap fruit. The physicochemical characteristics of the Brazilian genipap are presented in Table 1.

Blue colors are present in nature. Nevertheless, the applications for pigments from natural sources in food and beverages are scarce. Although genipap be a good source for obtaining blue color, the extraction of blue colorants from this source is a big challenge for modern science because this process has not been attempted often. There is a scarcity of natural blue metabolites compared to metabolites of other colors, and it is undeniable how useful a natural blue colorant would be for the food industry. This color is used in many foods, including candy, ice cream, condiments, beverages, chocolates, candy, gums, jellies, toppings and cakes.

The pH of the unripe genipap promotes the development of blue pigment in both the endocarp and mesocarp. The acidic pH (approximately 3.0) of the ripe fruit hinders the formation of blue pigments because an acidic medium prevents the reaction between the amine group with genipin, which leads to the formation of a secondary amide and results in a polymer with low molecular mass [44]. The higher the molecular mass, the greater the tinctorial strength of the blue pigments will be [44, 56]. Another factor that influences the formation of blue pigments is the presence of proteins. Since the endocarp has a greater protein content than the mesocarp, that part of the fruit also has a greater amount of blue pigments. According to Bentes *et al* [44], the protein content in the endocarp is 5 times higher than the mesocarp.

4.1. Chemical Characteristics of Genipap Fruit

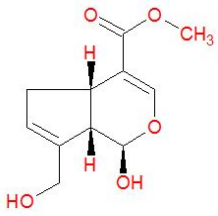
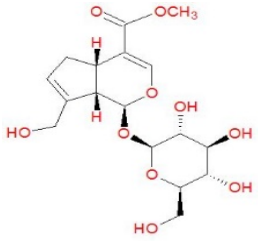
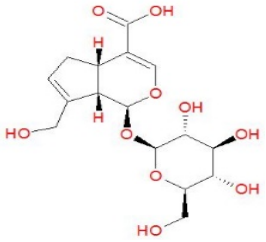
In terms of chemical composition, genipap is characterized by the presence of three iridoids: genipin, geniposide and geniposidic acid [57]. The iridoids are secondary metabolites usually found in many plants, especially as glycosides. Structurally, they are bicyclic monoterpenes (C10) with a basic skeleton that is a cyclopentane-[C]-pyran ring fused with a six-membered heterocycle oxygenate [58, 59]. Table 2 shows the basic structures of iridoids present in genipap and its main characteristics.

Table 1. Physicochemical characteristics of genipap

Physical and chemical characteristics	References					
	[10]	[52]	[53]	[54]	[55]	[44]
Moisture (%)	75.0 ± 0.2	74.7	80.4 ± 0.3	70.0 ± 0.1	93.5 ± 0.5	68.0 ± 1.4
pH	nq	4.2	3.9 ± 0.3	4	3.18 ± 0.02	5.21 ± 0.01
Protein (%)	0.67 ± 0.02	0.7	1.6 ± 0.1	0.5 ± 0.0	0.21 ± 0.01	3.2 ± 0.1
Lipids (%)	1.6 ± 0.2	0.3	1.6 ± 0.1	0.0 ± 0.0	0.34 ± 0.01	0.5 ±0 .1
Ash (%)	2.2 ± 0.3	1.0	0.7 ± 0.0	1.1 ± 0.1	0.4 ± 0.1	0.9 ± 0.1
Carbohydrates (%)	20.5	nq	14.6 ± 0.3	22.1 ± 0.5	4.4 ± 0.4	nq
Energy value	99 kcal/415kJ	nq	77.0 ±0.2 kcal/100g	91 ± 2 kcal/100g	22 ± 1 kcal	43.5 ± 1.3 kcal/100g
Ripe stage	Ripe	Unripe	Ripe	nm	nm	Unripe

nq, not quantified; nm, not mentioned.

Table 2. Chemical and physical characteristics of iridoids from genipap fruit

Chemical and physical characteristics	Genipin	Geniposide	Geniposidic acid
Structure			
Molecular formula	$C_{11}H_{14}O_5$	$C_{17}H_{24}O_{10}$	$C_{16}H_{22}O_{10}$
Molecular mass (g / mol)	226.23	388.37	374.34
Solubility	Water, methanol, ethanol, diethyl ether, propylene glycol	Water, methanol, ethanol	Water, ethanol, methanol
Melting point (°C)	120 - 121	163 - 164	133 - 136
Boiling point (°C)	416	641.4	684.12

Source: Bajaj [64]; Djerassi *et al* [47]; Guarnaccia *et al* [65]; Ozaki *et al* [60]; Ramos-de-la-Pena *et al* [66]; PubChem [67]

The geniposide is iridoid glycoside, which is often used in countries from Asia as a natural colorant. Furthermore, this plant is very traditional in Chinese culture due to its medicinal effects in the treatment of hepatics and inflammatory diseases [60, 61]. This iridoid is presented in genipap and constitutes approximately 4 to 6% of dry fruit [62]. There is little information with respect to the presence of geniposidic acid in the fruits of genipap, and the proportion of this iridoid in genipap fruits has not been reported in the literature yet. However, it is an iridoid of great interest to the pharmaceutical industry due to its antitumor effects [63].

Among the iridoids from genipap, genipin stands out for its ability to produce colorants [68]. Because of its applications as colorants, genipin has been widely studied because its coloring power is of great interest to the food, chemical and textile industries as well as its medicinal properties, which are of interest to the pharmaceutical industry [69]. Genipin is a colorless substance that was first isolated in 1960 by Djerassi *et al* [47]. When in contact with an epidermal protein, genipin produces a violet-blue color. This coloring effect has led to its widespread use in body paint among indigenous peoples [47]. Genipin reacts spontaneously to the presence of oxygen with primary amine groups of amino acids, peptides or proteins to form blue pigments [70]. These pigments have fluorescent properties optimized in the 590 nm excitation wavelength and with emission above 630 nm [71]. The genipin from genipap is present in proportions of 1-3 g / 100 g of fruit [72]. It is soluble in polar solvents, i.e., water, alcohol and propylene glycol, and it is stable in pH values ranging between 4.0-9.0 [66].

Genipin can be obtained directly from genipap using organic solvents or after enzymatic hydrolysis with β-glycosidases of geniposide from *Gardenia jasminoides* fruit [73, 74]. These pigments resulting from enzymatic hydrolysis of geniposide are more stable in alkaline medium (pH = 9.0) than in neutral (pH = 7.0) or acidic medium (pH = 5.0), and they remain stable after 10 hours at 60 - 90°C [73].

Brauch *et al* [11], compared the stability of the blue pigment obtained from genipap with blue pigments that are commonly used, including *Spirulina*, brilliant blue FCF (Blue no. 1), and indigo carmine (Blue no. 2). These authors observed that the blue pigments from genipap presented higher storage stability than Blue no. 2 and were less susceptible to an acidic pH (3.6) than *Spirulina*. At the end of their study, they concluded that these natural blue pigments are a promising alternative to synthetic colorants.

4.2. Methods for Genipin Extracting

The biggest challenge of using of genipap as colorant is the techniques for obtaining a high yield of genipin. Several technologies can be used to extract genipin, and selecting the best technology depends on the compound of interest, the available capital and the scale of production. Conventional technologies have already been used for years for obtaining bioactive compounds. However, it is currently necessary to use emerging technologies, which are environmental friendly and promote efficient extraction. Table 3 shows the main features of conventional and emerging technologies.

From the methods mentioned above the maceration, the extraction using high and low pressures and the extraction with ultrasound were used to obtain extracts rich in genipin. Although the traditional methods are low cost, they employ toxic solvents (except hydrodistillation) and long extraction times, which could favor the degradation of the genipin. The use of supercritical fluids is a recommended technology for the extraction of thermosensitive compounds [75]. However, it has not yet been investigated for the extraction of genipin. According to the characteristics of the genipin compound (item 4.1) it would be interesting to use supercritical fluid with the aid of a co-solvent, for instance

the ethanol, which in addition of being a safe solvent for food purposes allows the extraction of polar compounds. Further details of the conditions of genipin extraction by these methods are presented in item 4.2.1.

4.2.1. History of the Genipin Extraction

Attempts to obtain genipin from natural sources have been studied for decades. In 1994, Touyama *et al* [70] observed that in a hydroalcoholic medium under a nitrogen atmosphere, the reaction between genipin and methylamine (a simple primary amine) produced a yellow pigment and then a red-brown pigment, which changed to blue when reacting to oxygen. This blue pigment consists of a mixture of polymers with high molecular mass that are soluble in water, methanol, and ethanol [70]. In 1996, Penalber *et al* [87] used different organic solvents to extract blue pigments from genipap. In this study, the extracts obtained with water and ethanol resulted an intense blue colorant that becomes black at temperatures above 80°C. However, the use of hexane as solvent was not able to extract the dye, which may characterize the colorant a polar compound.

Table 3. Methods for genipin extraction

Traditional methods	**Characteristics**	**References**
Soxhlet	Requires a small amount of raw material; Low cost; Easy handling; Larger amount of solvents; High energy input; Long time for complete extraction; Use of toxic solvents; Presence of residual solvent in the extract.	[13, 76]
Maceration	Low cost; Easy handling; Larger amount of solvents; Long time for extraction; Use of toxic solvents.	[77]
Hydrodistillation	High energy input; Low cost; Easy handling; Long time for extraction; Use limited for thermally labile compounds; Presence of residual solvent in the extract; Partial hydrolysis of water sensitive compounds.	[78, 79]
Emerging Methods	**Characteristics**	**References**
Pressurized liquid extraction (PLE)	Large ranges of temperature (313 - 473 K) and pressure (3.5 to 35 MPa); Greater diffusion and mass transfer between the solute and the solvent; Selective method; Requires a small amount of solvent; Short time for extraction; Easy handling; Green solvents.	[80-82]
Supercritical fluid extraction (SFE)	Pressures and temperatures above the critical points of a compound or mixture; Selective method; Greater diffusion and mass transfer between the solute and the solvent; Short time for extraction; Green solvents.	[83, 84]
Ultrasound-assisted extraction	Powerful tool to accelerate analytical processes; Increase extraction yields; Combination of pressure, heat and turbulence for accelerates the mass transfer Short time; High reproducibility; Low solvent consumption; Operational simplicity High purity of the final product.	[80, 85, 86]

Five years after, Paik *et al* [88] studied the stability of blue pigments obtained by mechanical extraction of the dried fruits of *Gardenia jasminoides*. In this study, the pigments were formed from the reaction of genipin aglycone with the amino acids glycine, lysine, and phenylalanine. The experiment was performed under different pH (5.0, 7.0 and 9.0), temperature (60, 70, 80 and 90°C) and light intensity conditions (5000, 10000 and 20000 lux). Among the amino acids used lysine generated the largest remaining percentage of blue pigments after 10 h at 60°C. At pH 5.0, 7.0 and 9.0, the percentage of remaining pigments was 104, 102 and 110%, respectively. This outcome indicates that the amino group of lysine plays a crucial role in the formation of blue pigments.

Unripe fruits of genipap were subjected to mechanical extraction in the presence of water and aqueous ethanol at 50% and 95% by Renhe *et al* [12]. These authors assessed extraction at different pH values (4, 5, 6, 7, 8 and 9) and different temperatures (35, 45, 55, 65 and 75°C) at a ratio of 1:2 (a part of the fruit to two parts solvent). The extracts that were obtained were analyzed by colorimetry, and it was concluded that the temperature contributed to the formation of blue color. By increasing the temperature, the extract acquired a black color. The optimal conditions of the extraction with water were at 55°C and pH 4.0. The ethanol solutions had better performances at a temperature of 75°C and pH 4.0.

Processes for obtaining and applying blue colorants from genipap have already been patented by some authors. Wu *et al* [89] patented a method (US20090246343 A1, publication date in Oct. 1st, 2009) for producing natural stable color products by adding some edible materials to the juice of genipap. In this study, the authors used the ripe fruits of genipap and different shades of blue, green and purple were observed as well as the brown and black colors. The products generated in this experiment showed excellent stability under acidity and heat, which allowed the products to be used in food, beverages, medicines, dietary supplements, cosmetics, personal hygiene materials and animal feed.

A process for obtaining blue color was also patented by Echeverry *et al* [90] (US7927637 B2, publication date in Apr. 19th, 2011). In this study, the pulp was separated from the fruit and subsequently milled. Afterwards, the raw liquid juice was mixed with glycine. This mixture (juice and glycine) was heated for 2 hours at approximately 70°C. Then the extract was dehydrated using a lyophilization to produce a solid blue colorant.

Color compounds were isolated from the reaction of genipin from the genipap fruit with glycine. This study was patented with the number US20130345427 A1 and published in Dec. 2013. The aim of this research was to study the molecular structure of the blue pigment resulting from the reaction. The unripe fruit of genipap was freeze-dried and extracted by Soxhlet with dichloromethane. After extraction, the solvent was removed and genipin was identified by thin layer chromatography. Then glycine was dissolved in an aqueous medium at 70°C. A solution of genipin and methanol was added to this mixture and stirred for 4 hours. After the reaction, the mixture was lyophilized, and the blue powder was extracted with ethyl acetate to remove excess genipin and other polar compounds. Finally, the fractionation was performed by chromatography analysis of the materials resulting from the reaction [91].

Wu and Horn [92] patented a method (US8945640 B2, publication date in Feb. 3th, 2015) of producing extracts rich in genipin from genipap. The extraction developed by these authors involved the use of aqueous solvents (polar) and organic solvents (non-polar). First, the fruits were washed and then peeled. Water was used as a solvent and the mash that was obtained from the mixture (solvent + fruit) was filtered on a filter press to separate the solids. The pH was adjusted to 3.8-4.0, and the extract was concentrated in vacuum rotaevaporator. A second extraction with non-polar solvent was performed. The organic solvent was separated from the aqueous phase by decantation, and the organic phase was separated using a high speed centrifuged. The solvent was removed by evaporation and a solid extract rich in genipin was obtained (70% w / w). To obtain the colorant, the authors evaluated the use of the amino acids L-threonine, L-isoleucine, and L-histidine in the ex-tracts and observed that after heating, the amino acids L-threonine and L-isoleucine generated a green color, while the blue color was formed when L-histidine was added. The addition of L-alanine and xylose provided an extract with a red-orange color for the extract.

Most of the studies are limited to color analysis. Only in recent years have some studies examined the extraction conditions of the process and extraction yield.

Genipin was obtained from genipap by solid-liquid extraction by maceration of unripe fruits using chloroform at ratio of 1:2 (a part of the fruit to two parts of solvent). The yield of genipin obtained from unripe fruits stored on refrigeration (T < 0°C) for 41 days was 0.44 ± 0.06%, a yield 15 times higher than that obtained using freshly collected unripe fruits. These authors in their experiment observed color changes, where the extracts from the fresh fruits were greenish-white color while the extracts from fruits stored for 41 days were blue [69].

Obtaining genipin with ultrasound treatment was studied by Ramos-de-la-Pena *et al* [66]. In this study, the samples of genipap were submitted to temperatures of 5, 10 and 15ºC for 5, 10 and 15 minutes (285W, 24 kHz). The results obtained after cold-extraction showed that the process performed at 10°C for 15 min was the most efficient in terms of the yield of non-crosslinked genipin (7.9 ± 0.3 mg / g of the fruit).

Ramos-de-la-Peña *et al* [74] studied the recovery of genipin from genipap by high pressure processes combined with enzymatic treatments. Among the tested conditions, the pressure of 130 MPa provided the highest yield at the temperature of 9.3 ± 0.5°C without the addition of pectic enzymes. The yield obtained at these conditions was 34 ± 2 mg / g of fruit.

The genipin extraction with pressurized ethanol was studied by Náthia-Neves *et al* [93] in several parts of the

unripe genipap fruit. In this study, the authors observed that the endocarp presented with the highest recovery of genipin (48.6 ± 0.6 mg/g raw material) at 80°C and 12 bar.

5. Benefits to Human Health

As stated before, the geniposide releases aglycone genipin after hydrolytic cleavage by a β-deglycosidase enzyme in the human intestine. This iridoid has some pharmacological effects, such as activity against oxidative damage and inhibition of tumors [94]. In addition, several authors have called attention to the biological properties of genipin since this compound is able to act as an antimicrobial and anti-inflammatory agent [95, 96] in addition to having antilipoperoxidative [95] anti-cancer [97] anti-diabetic [98, 99], and antioxidant activity [100] as well as protecting against liver (hepatic) diseases [101] and protecting hippocampal neurons [102, 103]. These compounds also have antithrombotic [104] and neuroprotective effects [105, 106].

6. Conclusions

At the end of this review, it could be concluded that it is possible to obtain stable natural blue colorant using unripe fruits of genipap as a raw material. Although there are some limitations, the use of natural colorants has been increasingly encouraged due to health benefits and the quality of the final product. There are few studies regarding the yield of genipin from genipap fruit extraction. Additionally, there are no reports regarding the economic assessment of implementing this compound at an industrial scale. The lack of studies involving the extraction and application of genipin as a colorant agent can be related to the fact that using this agent in food is permitted only in some countries.

However, further investigations should be carried out before these colorants are used in industrial applications, among which should be:

- ✓ Develop more in vitro studies to ensure that the colorants obtained from genipap present no risk to human health;
- ✓ Use the colorants in different products and evaluate their stability and sensory quality at all stages of the production chain (i.e., production, transportation and marketing);
- ✓ Develop processes that allow blue pigments to be obtained with high purity and yield for the product to be competitive in the global market and ensure the processes are environmental friendly.

ACKNOWLEDGEMENTS

G. Náthia-Neves thanks CAPES/DEA/PROEX for Ph.D. assistantship and M. A. A. Meireles thanks CNPq for the productivity grant (302423/2015-0).

REFERENCES

[1] M. Mirjalili, K. Nazarpoor, L. Karimi, Eco-friendly dyeing of wool using natural dye from weld as co-partner with synthetic dye, Journal of Cleaner Production, 19 (2011) 1045-1051.

[2] L. Perez-Ibarbia, T. Majdanski, S. Schubert, N. Windhab, U.S. Schubert, Safety and regulatory review of dyes commonly used as excipients in pharmaceutical and nutraceutical applications, European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences, 93 (2016) 264-273.

[3] A.G. Newsome, C.A. Culver, R.B. Van Breemen, Nature's palette: the search for natural blue colorants, J Agric Food Chem, 62 (2014) 6498-6511.

[4] P. Amchova, H. Kotolova, J. Ruda-Kucerova, Health safety issues of synthetic food colorants, Regulatory toxicology and pharmacology: RTP, 73 (2015) 914-922.

[5] K. Golka, S. Kopps, Z.W. Myslak, Carcinogenicity of azo colorants: influence of solubility and bioavailability, Toxicology letters, 151 (2004) 203-210.

[6] FDA, Food and Drug Administration - Color Additives: FDA's Regulatory Process and Historical Perspectives, in, 2018.

[7] T. Taham, F.A. Cabral, M.A.S. Barrozo, Extraction of bixin from annatto seeds using combined technologies, The Journal of Supercritical Fluids, 100 (2015) 175-183.

[8] S.-B. Wu, C. Long, E.J. Kennelly, Phytochemistry and health benefits of jaboticaba, an emerging fruit crop from Brazil, Food Research International, 54 (2013) 148-159.

[9] M. Valizadeh Kiamahalleh, G. Najafpour-Darzi, M. Rahimnejad, A.A. Moghadamnia, M. Valizadeh Kiamahalleh, High performance curcumin subcritical water extraction from turmeric (Curcuma longa L.), Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 1022 (2016) 191-198.

[10] R.G.C.L. Porto, B.V.S. Cardoso, N.V.d.A. Barros, E.M.F. Cunha, M.A.d.M. Araújo, R.S.d.R. Moreira-Araújo, Chemical Composition and Antioxidant Activity of Genipa Americana L. (Jenipapo) of the Brazilian Cerrado, Journal of Agriculture and Environmental Sciences, 3 (2014).

[11] J.E. Brauch, S.P. Zapata-Porras, M. Buchweitz, J.K. Aschoff, R. Carle, Jagua blue derived from Genipa americana L. fruit: A natural alternative to commonly used blue food colorants?, Food Research International, (2016).

[12] I.R.T. Renhe, P.C. Stringheta, F.F.e. Silva, T.V.d. Oliveira, Obtenção de corante natural azul extraído de frutos de jenipapo, Pesquisa Agropecuária Brasileira, 44 (2009) 649-652.

[13] J. Azmir, I.S.M. Zaidul, M.M. Rahman, K.M. Sharif, A. Mohamed, F. Sahena, M.H.A. Jahurul, K. Ghafoor, N.A.N. Norulaini, A.K.M. Omar, Techniques for extraction of bioactive compounds from plant materials: A review, Journal of Food Engineering, 117 (2013) 426-436.

[14] P. Vazquez-Roig, Y. Picó, Pressurized liquid extraction of

organic contaminants in environmental and food samples, TrAC Trends in Analytical Chemistry, 71 (2015) 55-64.

[15] Brasil, BRASIL - Decreto nº 55871, de 26 de março de 1965. Modifica o Decreto nº 50.040, de 24 de janeiro de 1961, referente a normas reguladoras do emprêgo de aditivos para alimentos, (1965).

[16] Brasil, BRASIL - Decreto nº 50040, de 24 de janeiro de 1961. Dispõe sobre Normas Técnicas Especiais Reguladoras do Emprego de Aditivos Químicos a Alimentos, (1961).

[17] ANVISA, ANVISA – Agência Nacional de Vigilância Sanitaria. Resolução - CNNPA nº 37, de 1977., (1977).

[18] ANVISA, ANVISA – Agência Nacional de Vigilância Sanitaria. Resolução - CNNPA/MSl nº 44, de 1977. Estabelece as condições gerais de elaboração, classificação, apresentação, designação, composição e fatores essenciais de qualidade dos corantes empregados na produção de alimentos (e bebidas). (1977).

[19] ANVISA, ANVISA – Agência Nacional de Vigilância Sanitaria. RDC nº 259, de 2002. Aprova o Regulamento Técnico sobre Rotulagem de Alimentos Embalados., (2002).

[20] ANVISA, ANVISA – Agência Nacional de Vigilância Sanitaria. RDC nº 340, de 2002. Estabelece que as empresas fabricantes de alimentos que contenham na sua composição o corante tartrazina (INS 102) devem obrigatoriamente declarar na rotulagem, na lista de ingredientes, o nome do corante tartrazina por extenso., (2002).

[21] ANVISA, ANVISA – Agência Nacional de Vigilância Sanitaria. Informe Técnico nº 68, de 3 de setembro de 2015. Classificação dos corantes caramelos II, III e IV e dos demais corantes autorizados para uso em alimentos., (2015).

[22] S. Lehto, M. Buchweitz, A. Klimm, R. Straßburger, C. Bechtold, F. Ulberth, Comparison of food colour regulations in the EU and the US: a review of current provisions, Food Additives & Contaminants: Part A, 34 (2017) 335-355.

[23] EU, Official Journal of the European Union - Commission Directive 95/45/EC of 26 July 1995. Laying down specific purity criteria concerning colours for use in foodstuffs, (1995).

[24] FDA, Food and Drugs Administration - Summary of Color Additives for Use in the United States in Foods, Drugs, Cosmetics, and Medical Devices, (2016).

[25] J. König, 2 - Food colour additives of synthetic origin, in: M.J. Scotter (Ed.) Colour Additives for Foods and Beverages, Woodhead Publishing, Oxford, 2015, pp. 35-60.

[26] D.B. Rodriguez-Amaya, Natural food pigments and colorants, Current Opinion in Food Science, 7 (2016) 20-26.

[27] N. Martins, C.L. Roriz, P. Morales, L. Barros, I.C.F.R. Ferreira, Food colorants: Challenges, opportunities and current desires of agro-industries to ensure consumer expectations and regulatory practices, Trends in Food Science & Technology, 52 (2016) 1-15.

[28] G.L. Dotto, L.A. Pinto, M.A. Hachicha, S. Knani, New physicochemical interpretations for the adsorption of food dyes on chitosan films using statistical physics treatment, Food chemistry, 171 (2015) 1-7.

[29] K. Yamjala, M.S. Nainar, N.R. Ramisetti, Methods for the analysis of azo dyes employed in food industry - A review, Food chemistry, 192 (2016) 813-824.

[30] A. Gürses, M. Açıkyıldız, K. Güneş, M.S. Gürses, Dyes and Pigments: Their Structure and Properties, in: Dyes and Pigments, Springer International Publishing, Cham, 2016, pp. 13-29.

[31] M. Shahid, I. Shahid ul, F. Mohammad, Recent advancements in natural dye applications: a review, Journal of Cleaner Production, 53 (2013) 310-331.

[32] R.M. Selvam, G. Athinarayanan, A.U.R. Nanthini, A.J.A.R. Singh, K. Kalirajan, P.M. Selvakumar, Extraction of natural dyes from Curcuma longa, Trigonella foenum graecum and Nerium oleander, plants and their application in antimicrobial fabric, Industrial Crops and Products, 70 (2015) 84-90.

[33] M.R. Narayan, Review: Dye sensitized solar cells based on natural photosensitizers, Renewable and Sustainable Energy Reviews, (2011).

[34] R.K. Saini, S.H. Nile, S.W. Park, Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities, Food Research International, 76 (2015) 735-750.

[35] E. Fernández-García, I. Carvajal-Lérida, M. Jarén-Galán, J. Garrido-Fernández, A. Pérez-Gálvez, D. Hornero-Méndez, Carotenoids bioavailability from foods: From plant pigments to efficient biological activities, Food Research International, 46 (2012) 438-450.

[36] G. Jing, T. Li, H. Qu, Z. Yun, Y. Jia, X. Zheng, Y. Jiang, Carotenoids and volatile profiles of yellow- and red-fleshed papaya fruit in relation to the expression of carotenoid cleavage dioxygenase genes, Postharvest Biology and Technology, 109 (2015) 114-119.

[37] M. Carocho, P. Morales, I.C.F.R. Ferreira, Natural food additives: Quo vadis?, Trends in Food Science & Technology, 45 (2015) 284-295.

[38] L.M. Rodrigues, S.C. Alcázar-Alay, A.J. Petenate, M.A.A. Meireles, Bixin extraction from defatted annatto seeds, Comptes Rendus Chimie, 17 (2014) 268-283.

[39] O. Safari, M.M.S. Atash, The effects of dietary supplement of annatto (Bixa orellana) seed meal on blood carotenoid content and fillet color stability in rainbow trout (Oncorhynchus mykiss), Aquaculture, 437 (2015) 275-281.

[40] D.T. Santos, J.Q. Albarelli, M.M. Beppu, M.A.A. Meireles, Stabilization of anthocyanin extract from jabuticaba skins by encapsulation using supercritical CO2 as solvent, Food Research International, 50 (2013) 617-624.

[41] F.C. Stintzing, R. Carle, Betalains – emerging prospects for food scientists, Trends in Food Science & Technology, 18 (2007) 514-525.

[42] A. Gengatharan, G.A. Dykes, W.S. Choo, Betalains: Natural plant pigments with potential application in functional foods, LWT - Food Science and Technology, 64 (2015) 645-649.

[43] G. Jain, K.S. Gould, Are betalain pigments the functional homologues of anthocyanins in plants? Environmental and Experimental Botany, 119 (2015) 48-53.

[44] A.d.S. Bentes, H.A.L. de Souza, J. Amaya-Farfan, A.S. Lopes, L.J.G. de Faria, Influence of the composition of unripe

genipap (Genipa americana L.) fruit on the formation of blue pigment, J Food Sci Technol, 52 (2015) 3919-3924.

[45] A.M. Ramos-de-la-Peña, C.M.G.C. Renard, J. Montañez, M. de la Luz Reyes-Vega, J.C. Contreras-Esquivel, A review through recovery, purification and identification of genipin, Phytochemistry Reviews, 15 (2016) 37-49.

[46] FDA, - Food and Drugs Administration - Listing of color additives exempt from certification; food, drug and cosmetic labeling: cochineal extract and carmine declaration (21 CFR parts 73 and 101). Federal register 74 (2), 74 (2), (2009) 207-217.

[47] C. Djerassi, J.D. Gray, F.A. Kincl, Naturally Occurring Oxygen Heterocyclics. IX.1Isolation and Characterization of Genipin 2, The Journal of Organic Chemistry, 25 (1960) 2174-2177.

[48] J. Pino, R. Marbot, C. Vazquez, Volatile constituents of genipap (Genipa americana L.) fruit from Cuba, Flavour and Fragrance Journal, 20 (2005) 583-586.

[49] G.T. Prance, FRUITS OF TROPICAL CLIMATES | Fruits of Central and South America, in: B. Caballero (Ed.) Encyclopedia of Food Sciences and Nutrition (Second Edition), Academic Press, Oxford, 2003, pp. 2810-2816.

[50] M.P. Corrêa, L.A. Pena, Dicionário das plantas úteis do Brasil e das exóticas cultivadas, Rio de Janeiro: Ministério da Agricultura, Instituto Brasileiro de Desenvolvimento Florestal, 1984.

[51] A.V.C. Silva, K.C.S. Freire, A.d.S. Lédo, A.R.C. Rabbani, Diversity and genetic structure of jenipapo (Genipa americana L.) Brazilian accessions, Scientia Agricola, 71 (2014) 387-393.

[52] R.W. Figueiredo, G.A. Maia, L.F.F. Holanda, J.C. Monteiro, Características físicas e químicas do jenipapo, Pesquisa Agropecuária Brasileira, 21 (1986).

[53] F.R. Hamacek, A.V.B. Moreira, H.S.D. Martino, S.M.R. Ribeiro, H.M. Pinheiro-Sant'Ana, Valor nutricional, caracterização física e físico-química de jenipapo (Genipa americana L.) do cerrado de Minas Gerais, Alimentos e Nutrição Araraquara, 24 (2013) 78.

[54] P. Pacheco, J.G. Da Paz, C.O. Da Silva, G.B. Pascoal, COMPOSIÇÃO CENTESIMAL, COMPOSTOS BIOATIVOS E PARÂMETROS FÍSICO-QUÍMICOS DO JENIPAPO (Genipa americana L.) IN NATURA, DEMETRA: Alimentação, Nutrição & Saúde, 9 (2014).

[55] V.R. Souza, P.A.P. Pereira, F. Queiroz, S.V. Borges, J. de Deus Souza Carneiro, Determination of bioactive compounds, antioxidant activity and chemical composition of Cerrado Brazilian fruits, Food chemistry, 134 (2012) 381-386.

[56] J.-E. Park, J.-Y. Lee, H.-G. Kim, T.-R. Hahn, Y.-S. Paik, Isolation and Characterization of Water-Soluble Intermediates of Blue Pigments Transformed from Geniposide of Gardenia jasminoides, Journal of Agricultural and Food Chemistry, 50 (2002) 6511-6514.

[57] M. Ono, M. Ueno, C. Masuoka, T. Ikeda, T. Nohara, Iridoid glucosides from the fruit of Genipa americana, Chemical and pharmaceutical bulletin, 53 (2005) 1342-1344.

[58] A. Bianco, Recent developments in iridoids chemistry, Pure and applied chemistry, 66 (1994) 2335-2338.

[59] B. Dinda, S. Debnath, Y. Harigaya, Naturally occurring iridoids. A review, Part 1, Chemical and pharmaceutical bulletin, 55 (2007) 159-222.

[60] A. Ozaki, M. Kitano, N. Furusawa, H. Yamaguchi, K. Kuroda, G. Endo, Genotoxicity of gardenia yellow and its components, Food and Chemical Toxicology, 40 (2002) 1603-1610.

[61] I.H. Pan, H.-H. Chiu, C.-H. Lu, L.-T. Lee, Y.-K. Li, Aqueous two-phase extraction as an effective tool for isolation of geniposide from gardenia fruit, Journal of Chromatography A, 977 (2002) 239-246.

[62] M.F. Butler, Y.-F. Ng, P.D.A. Pudney, Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin, Journal of Polymer Science Part A: Polymer Chemistry, 41 (2003) 3941-3953.

[63] H.-Y. Hsu, J.-J. Yang, S.-Y. Lin, C.-C. Lin, Comparisons of geniposidic acid and geniposide on antitumor and radioprotection after sublethal irradiation, Cancer Letters, 113 (1997) 31-37.

[64] Y.P.S. Bajaj, Medicinal and Aromatic Plants IV, Springer Berlin Heidelberg, 2012.

[65] R. Guarnaccia, K.M. Madyastha, E. Tegtmeyer, C.J. Coscia, Geniposidic acid, an iridoid glucoside from Genipa americana, Tetrahedron Letters, 13 (1972) 5125-5127.

[66] A.M. Ramos-de-la-Pena, C.M. Renard, L. Wicker, J.C. Montanez, L.A. Garcia-Cerda, J.C. Contreras-Esquivel, Environmental friendly cold-mechanical/sonic enzymatic assisted extraction of genipin from genipap (Genipa americana), Ultrasonics sonochemistry, 21 (2014) 43-49.

[67] PubChem, Compound Database, in, https://pubchem.ncbi.nlm.nih.gov/search/search.cgi, 2018.

[68] G.N. NÁTHIA-NEVES, Gislaine; VARDANEGA, Renata; MEIRELES, Maria Angela de Almeida, Identification and quantification of genipin and geniposide from Genipa americana L. by HPLC-DAD using a fused-core column, Food Science and Technology (Campinas), (2018). (doi: 10.1590/1678-457X.17317).

[69] C.L. Velásquez, A. Rivas, I.S. Ocanto, Obtención de Genipina a partir de frutos de caruto (Genipa americana L.) del llano venezolano, Avances en Química, 9 (2014) 75-86.

[70] R. Touyama, Y. Takeda, K. Inoue, I. Kawamura, M. Yatsuzuka, T. Ikumoto, T. Shingu, T. Yokoi, H. Inouye, Studies on the Blue Pigments Produced from Genipin and Methylamine. I. Structures of the Brownish-Red Pigments, Intermediates Leading to the Blue Pigments, CHEMICAL & PHARMACEUTICAL BULLETIN, 42 (1994) 668-673.

[71] P. Thomas, K. Farrugia, An investigation into the enhancement of fingermarks in blood on paper with genipin and lawsone, Science & justice: journal of the Forensic Science Society, 53 (2013) 315-320.

[72] A.M. Ramos-de-la-Pena, J.C. Montanez, L. Reyes-Vega Mde, J.C. Contreras-Esquivel, Temperature model for process impact non-uniformity in genipin recovery by high pressure processing, Food chemistry, 187 (2015) 444-450.

[73] S.-W. Lee, J.-M. Lim, S.-H. Bhoo, Y.-S. Paik, T.-R. Hahn, Colorimetric determination of amino acids using genipin from Gardenia jasminoides, Analytica Chimica Acta, 480

(2003) 267-274.

[74] A.M. Ramos-de-la-Peña, J.C. Montañez, M.d.l.L. Reyes-Vega, M.E. Hendrickx, J.C. Contreras-Esquivel, Recovery of genipin from genipap fruit by high pressure processing, LWT - Food Science and Technology, 63 (2015) 1347-1350.

[75] I. Lepojević, Ž. Lepojević, B. Pavlić, M. Ristić, Z. Zeković, S. Vidović, Solid-liquid and high-pressure (liquid and supercritical carbon dioxide) extraction of Echinacea purpurea L, The Journal of Supercritical Fluids, 119 (2017) 159-168.

[76] M.D. Luque de Castro, F. Priego-Capote, Soxhlet extraction: Past and present panacea, Journal of chromatography. A, 1217 (2010) 2383-2389.

[77] J. Duval, V. Pecher, M. Poujol, E. Lesellier, Research advances for the extraction, analysis and uses of anthraquinones: A review, Industrial Crops and Products, 94 (2016) 812-833.

[78] F. Chemat, M.A. Vian, G. Cravotto, Green Extraction of Natural Products: Concept and Principles, International journal of molecular sciences, 13 (2012) 8615-8627.

[79] S. Zhao, D. Zhang, Supercritical CO2 extraction of Eucalyptus leaves oil and comparison with Soxhlet extraction and hydro-distillation methods, Separation and Purification Technology, 133 (2014) 443-451.

[80] P.V. Gadkari, M. Balaraman, Catechins: Sources, extraction and encapsulation: A review, Food and Bioproducts Processing, 93 (2015) 122-138.

[81] A. Mustafa, C. Turner, Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review, Anal Chim Acta, 703 (2011) 8-18.

[82] J.F. Osorio-Tobón, M.A.A. Meireles, Recent Applications of Pressurized Fluid Extraction: Curcuminoids Extraction with Pressurized Liquids, Food and Public Health, 3 (2013) 289-303.

[83] K.M. Sharif, M.M. Rahman, J. Azmir, A. Mohamed, M.H.A. Jahurul, F. Sahena, I.S.M. Zaidul, Experimental design of supercritical fluid extraction – A review, Journal of Food Engineering, 124 (2014) 105-116.

[84] G.L. Zabot, M.N. Moraes, M.A.A. Meireles, Supercritical Technology Applied to the Production of Bioactive Compounds: Research Studies Conducted at LASEFI from 2009 to 2013, Food and Public Health, 4 (2014) 36-48.

[85] F. Chemat, N. Rombaut, A.-G. Sicaire, A. Meullemiestre, A.-S. Fabiano-Tixier, M. Abert-Vian, Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review, Ultrasonics sonochemistry, 34 (2017) 540-560.

[86] R. Vardanega, D.T. Santos, M.A. Meireles, Intensification of bioactive compounds extraction from medicinal plants using ultrasonic irradiation, Pharmacognosy reviews, 8 (2014) 88-95.

[87] T.d.A. Penalber, M. Sadala, M. Castro, L.d. Faria, Ensaios extração e aplicação corantes do fruto jenipapeiro (Genipa americana), Revista Brasileira de Corantes Naturais, 2 (1996) 129-135.

[88] Y.-S. Paik, C.-M. Lee, M.-H. Cho, T.-R. Hahn, Physical Stability of the Blue Pigments Formed from Geniposide of Gardenia Fruits: Effects of pH, Temperature, and Light, Journal of Agricultural and Food Chemistry, 49 (2001) 430-432.

[89] S. Wu, C. Ford, G. Horn, Stable Natural Color Process, Products and Use Thereof, in, Google Patents, 2009.

[90] L.F. Echeverry, S.P. Zapata, L.F. Torres, Blue colorant derived from Genipa americana fruit, in, Google Patents, 2011.

[91] L.F. Echeverry, J.F. Gil, E. Vargas, Colorant compound derived from genipa americana genipin and glycine, in, Google Patents, 2012.

[92] S. Wu, G. Horn, Genipin-rich material and its use, in, Google Patents, 2015.

[93] G. Náthia-Neves, A.G. Tarone, M.M. Tosi, M.R. Maróstica Júnior, M.A.A. Meireles, Extraction of bioactive compounds from genipap (Genipa americana L.) by pressurized ethanol: Iridoids, phenolic content and antioxidant activity, Food Research International, 102 (2017) 595-604.

[94] M. Buchweitz, Natural Solutions for Blue Colors in Food, Handbook on Natural Pigments in Food and Beverages: Industrial Applications for Improving Food Color, (2016) 355.

[95] H.-J. Koo, Y.S. Song, H.-J. Kim, Y.-H. Lee, S.-M. Hong, S.-J. Kim, B.-C. Kim, C. Jin, C.-J. Lim, E.-H. Park, Antiinflammatory effects of genipin, an active principle of gardenia, European Journal of Pharmacology, 495 (2004) 201-208.

[96] H.J. Koo, K.H. Lim, H.J. Jung, E.H. Park, Anti-inflammatory evaluation of gardenia extract, geniposide and genipin, Journal of ethnopharmacology, 103 (2006) 496-500.

[97] B.C. Kim, H.G. Kim, S.A. Lee, S. Lim, E.H. Park, S.J. Kim, C.J. Lim, Genipin-induced apoptosis in hepatoma cells is mediated by reactive oxygen species/c-Jun NH2-terminal kinase-dependent activation of mitochondrial pathway, Biochemical pharmacology, 70 (2005) 1398-1407.

[98] X.-l. Shen, H. Liu, H. Xiang, X.-m. Qin, G.-h. Du, J.-s. Tian, Combining biochemical with 1H NMR-based metabolomics approach unravels the antidiabetic activity of genipin and its possible mechanism, Journal of Pharmaceutical and Biomedical Analysis, (2016).

[99] T. Ling-hu, X.-l. Shen, J.-s. Tian, X.-m. Qin, Investigation on Endogenous Metabolites in Pancreas of Diabetic Rats after Treatment by Genipin through 1H-NMR-based Metabolomic Profiles, Chinese Herbal Medicines, 8 (2016) 133-138.

[100] B.F. Juma, R.R.T. Majinda, Constituents of Gardenia volkensii: their brine shrimp lethality and DPPH radical scavenging properties, Natural product research, 21 (2007) 121-125.

[101] S.J. Kim, J.K. Kim, D.U. Lee, J.H. Kwak, S.M. Lee, Genipin protects lipopolysaccharide-induced apoptotic liver damage in D-galactosamine-sensitized mice, Eur J Pharmacol, 635 (2010) 188-193.

[102] M. Yamazaki, N. Sakura, K. Chiba, T. Mohri, Prevention of the Neurotoxicity of the Amyloid. BETA. Protein by

Genipin, Biological and Pharmaceutical Bulletin, 24 (2001) 1454-1455.

[103] J.-l. Chen, B.-y. Shi, H. Xiang, W.-j. Hou, X.-m. Qin, J.-s. Tian, G.-h. Du, 1H NMR-based metabolic profiling of liver in chronic unpredictable mild stress rats with genipin treatment, Journal of Pharmaceutical and Biomedical Analysis, 115 (2015) 150-158.

[104] Y. Suzuki, K. Kondo, Y. Ikeda, K. Umemura, Antithrombotic effect of geniposide and genipin in the mouse thrombosis model, Planta medica, 67 (2001) 807-810.

[105] R.H. Hughes, V.A. Silva, I. Ahmed, D.I. Shreiber, B. Morrison, Neuroprotection by genipin against reactive oxygen and reactive nitrogen species-mediated injury in organotypic hippocampal slice cultures, Brain Research, 1543 (2014) 308-314.

[106] M. Tanaka, M. Yamazaki, K. Chiba, Neuroprotective Action of Genipin on Tunicamycin-Induced Cytotoxicity in Neuro2a Cells, Biological and Pharmaceutical Bulletin, 32 (2009) 1220-1223.

- *CHAPTER 3* -

METHODOLOGY OF ANALYSIS OF GENIPIN AND GENIPOSIDE BY HPLC

Identification and quantification of genipin and geniposide from Genipa americana L. by HPLC-DAD using a fused-core column

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Article published in the journal Food Science and Technology (Campinas), 2018

ISSN 0101-2061. DDOI: 10.1590/1678-457X.17317

Received 18 May 2017, Accepted 15 October 2017

Article available in:

http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0101-20612018005003102

ISSN 0101-2061

DOI: http://dx.doi.org/10.1590/1678-457X.17317

Identification and quantification of genipin and geniposide from *Genipa americana* L. by HPLC-DAD using a fused-core column

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Abstract

In this work, it was developed a fast, simple and selective method for quantification of genipin and geniposide from unripe fruits of genipap, which are known as natural colorants, blue and yellow, respectively. The compounds separation was performed in a fused-core C18 column using as mobile phase water (A) and acetonitrile (B) both acidified with 0.1% formic acid, with the following gradient: 0 min, 99% A; 9 min, 75% A; 10 min, 99% A and 13 min, 99% A. The temperature and flow rate that allowed the best chromatographic performance were 35 °C and 1.5 mL/min, respectively, resulting a total run time of 13 min, including column clean-up and re-equilibration. This short analysis time represents an advantage compared to the methods reported in the literature where the running times are 2-5 times greater. The detection wavelength was set at 240 nm. The method validation was performed based on specificity, linearity, detection and quantification limits, precision and accuracy, according to ICH methodology. Finally, the developed method was suitable for monitoring analysis of those compounds content in vegetable samples.

Keywords: blue natural colorant; method validation; iridoids.

Practical Application: This method has a great potential to be used by the industry for analysis of genipin and geniposide.

1 Introduction

Genipap (*Genipa americana* L.) belongs to *Rubiacea* family and Genipa genus. It is a native plant from America, found mainly in Central and South regions of this continent (Djerassi et al., 1960; Ramos-de-la-Peña et al., 2015b). When in its ripe stage the genipap pulp is succulent, acidic and hard which is consumed mainly as juices, jams and liqueurs (Pino et al., 2005; Prance, 2003).

The unripe genipap fruit is rich in iridoids, which are secondary metabolites usually found in many plants, normally as glycosides. Structurally, iridoids are bicyclic monoterpenes (C10), whose basic skeleton is a cyclopentane-[C]-pyran ring typically fused with a six-membered heterocycle oxygenate (Bianco, 1994; Dinda et al., 2007). Among the iridoids present in the fruit, genipin and geniposide stand out as natural sources for obtaining the blue color (Velásquez et al., 2014).

The genipin is a colorless substance, present in unripe fruits of genipap, that is able to react spontaneously in the presence of oxygen, with primary amine groups of amino acids, peptides or proteins and form blue color (Djerassi et al., 1960). The genipin is present in *Genipa americana* L. in the proportion of 1-3% of fruit (Ramos-de-la-Peña et al., 2014). The genipin can be obtained directly from genipap by extraction with organic solvents or after enzymatic hydrolysis of geniposide with β-glycosidases (Ramos-de-la-Peña et al., 2015a; Thomas & Farrugia, 2013).

The geniposide is often used in Asian countries as a natural colorant and very traditional in Chinese culture for its medicinal effects in treating liver and inflammatory diseases. This iridoid represents about 4 to 6% of the dry fruit (Butler et al., 2003). The chemical structure of these compounds is shown in Figure 1.

Genipap has been used since ancient times by indigenous for body painting and nowadays it appears as an alternative for obtaining blue colorants for food and chemical industries (Ferreira, 2015). Currently, natural colorants applications have been greatly increased due to the interest for replacing synthetic additives by natural compounds. In addition to providing color, these compounds have biological activity against oxidative damage, inhibition of tumor and anti-inflammatory activities of great interest for pharmaceutical industry (Buchweitz, 2016; Koo et al., 2006).

Genipin and geniposide identification and quantification in real samples are mainly made by High-Performance Liquid Chromatography (HPLC). The major disadvantage of the existing methods is related to the analysis time. Some methods take between 35 and 75 minutes (Bentes & Mercadante, 2014; Bergonzi et al., 2012; Lee et al., 2014; Li et al., 2016; Wang et al., 2016) which limits its use on genipin and geniposide production scale.

Received 18 May, 2017
Accepted 15 Oct., 2017
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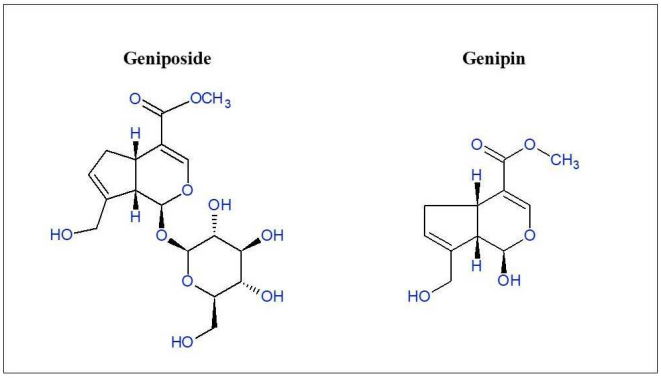

Figure 1. Chemical structure of geniposide and genipin.

To meet the demands for replacing synthetic by natural colorants, new methods to extract selectively these color additives have been developed. Therefore, it is interesting to develop also selective methods for the quantitation of these compounds. The aim of this study was to develop and to validate a reliable and fast HPLC method for simultaneous determination of genipin and geniposide from *Genipa americana* L. This method is helpful for natural colorants and pharmaceutical industries that use these iridoids in their formulations.

2 Materials and methods

2.1 Chemical and solvents

HPLC grade acetonitrile was purchased from Scharlau (Barcelona, Spain), formic acid and ethanol was obtained from Dinâmica (São Paulo, Brazil). Ultrapure water was supplied by a Milli-Q Advantage 8 Purifier System from Millipore (Bedford, USA). Genipin and geniposide standards (purity > 98%) were purchased from Sigma-Aldrich (St. Louis, USA).

2.2 Samples

The unripe genipap fruits were obtained from Sítio do Bello (Paraibuna, Brazil). The fruits were frozen with liquid nitrogen and stored in domestic freezer (-20 °C) until being processed for the extraction. The samples were prepared and extracted according to Náthia-Neves et al. (2017). The samples were extracted at 50 °C and 0.2 MPa with ethanol during 30 min. For each extraction assay 4 g of raw material and 20 g of solvent were used, resulting in a solvent to sample ratio of 5:1. After the extraction, the extracts were filtered through a 0.45 µm nylon seringe filter (Sinergia Cientifica, Campinas, Brazil) and diluted 5 times (200 µL of extract in 800 µL of solvent) to acetonitrile:water (1:1) for chromatographic analysis.

2.3 Chromatographic instrumentation

HPLC analysis was carried out on an Alliance 2695/2695D Separation Module (Waters, Milford, USA) with integrated column heater and auto-sampler and a photodiode array detector (2998, Waters, Milford, USA). Compounds separation was carried out on a fused-core C_{18} column (Kinetex, 100 × 4.6 mm i.d.; 2.6 µm; Phenomenex, Torrance, USA). The kinetic dead volume (V_m) of the column was 740 ± 5 µL and the extra-column volume was 62.5 ± 0.1 µL, as described in a previous study (Osorio-Tobón et al., 2016). The HPLC system dwell volume was described by the manufacturer as < 650 µL.

Chromatographic conditions development

The mesocarp ethanolic extract from genipap was the sample employed in all the chromatographic tests for the quantification of iridoids genipin and geniposide. The chromatographic conditions tested were the **mobile phase composition** consisted of water (acidified or not with formic acid 0.1% v/v, solvent A) and acetonitrile (acidified or not with formic acid 0.1%, v/v, solvent B), **temperatures** (30, 35 and 40 °C), **flow rates** (0.5, 1.0 and 1.5 mL/min) and **equilibration times** (1-5 min). UV spectra was monitored between 200 and 600 nm and the peaks of the iridoids were integrated at 240 nm.

Method validation

The method was validated according to ICH guidelines based on specificity, linearity and range, limits of detection and quantification, precision and accuracy (International Council for Harmonisation, 2005), with some adaptations to food material.

Specificity

The identification of iridoids present in the sample was achieved by the comparison of retention times and UV spectra of separated compounds with the authentic standard. Column efficiency was evaluated on basis of retention time, width, K prime, selectivity, symmetry factor, width at baseline and resolution of the peaks of iridoids geniposide and genipin. All performance parameters were calculated using the US Pharmacopeia (USP) option by the Empower 3 software.

Linearity and range

The stock solution of genipin standard was prepared by dissolving 25 mg of genipin in 10 mL of acetonitrile:water (1:1). The stock solution of geniposide standard was prepared by dissolving 10 mg of geniposide in 10 mL of acetonitrile:water (1:1). The curve of each iridoids was prepared in triplicate by plotting the concentration (0.1-1000 µg/mL for geniposide and 0.1-2500 µg/mL for genipin) against area of the peak. Regression equations and correlation coefficient (r^2) were calculated using OriginPro® v. 9.0 software.

Limits of detection and quantitation

The limit of detection (LoD) and limit of quantitation (LoQ) were determined by calculation of the signal-to-noise ratio. A signal-to-noise ratio of 3:1 was considered for estimating the LoD and the signal-to-noise ratio of 10:1 corresponded to the LoQ.

Precision and accuracy

The repeatability and intermediate precision of the developed method were evaluated in terms of peak area and retention time of the iridoids. A total of 30 HPLC analyses were performed

on three successive days (10 analyses per day) using the same sample, a mesocarp ethanolic extract from genipap.

The accuracy of the method was tested by the spiking/recovery technique. Firstly, three independent solutions of extract were prepared with the following iridoid concentrations: 28.65, 64.21 and 124.40 µg/mL. 200 µL of each solution were spiked with 3.86 µg of geniposide and 3.13 µg of genipin by adding 70 µL of each standard solution containing 55.15 µg/mL and 44.70 µg/mL of geniposide and genipin, respectively and each one was injected three times. The average percentage recovery was calculated for each level of concentration.

3 Results and discussion

3.1 Optimization of chromatographic conditions

The better overall peaks separation and resolution were obtained with the solvents acidified with formic acid. The reduction of pH of the mobile phase is commonly used for the separation of iridoids (Bentes & Mercadante, 2014; Bergonzi et al., 2012) as well as other bioactive compounds, such as curcurminoids (Osorio-Tobón et al., 2016), beta-ecdysone (Rostagno et al., 2014) and bixin (Chisté et al., 2011), among others.

The column temperature selected was 35 °C because a better resolution and reproducibility were obtained and it was below the maximum column operating temperature of 60 °C. The increase of temperature slightly decreased the retention time of iridoids. In the literature, temperatures between 25 and 30 °C were used for iridoids separation (Bentes & Mercadante, 2014; Bergonzi et al., 2012; Wang et al., 2016).

The mobile phase flow rate was increased step-by-step from 0.5 to 1.5 mL/min. Maintaining the temperature column at 35 °C, the retention time decreased 40% for the iridoids by increasing the flow rate. The separation of the iridoids was achieved in approximately 9 minutes, which is a short time for the separation of the compounds. Re-equilibration time is necessary in gradient HPLC to ensure that the column environment has returned to the initial stable conditions. These conditions are particularly important when using gradient elution because the difference between the initial and final organic composition of the mobile phase is significant (Zabot et al., 2014). It was necessary 4 min between runs to clean-up and return to the initial conditions of the method. The re-equilibration time represent 31% of the total run time, which was 13 minutes (including elution, clean-up and re-equilibration) and is equivalent to 9.4 volumes of the column.

3.2 Characteristics of the HPLC method

The optimized conditions of the chromatographic method consisted of the following gradient: 0 min, 99% A; 9 min, 75% A; 10 min, 99% A and 13 min, 99% A. The column was maintained at 35 °C, working with a flow rate of 1.5 mL/min and a re-equilibration time of 4 minutes. Representative chromatograms of the ethanolic extract of genipap and the iridoids standards are shown in Figure 2. Genipin and geniposide were identified through the retention times and maximum absorption wavelength.

The retention times of geniposide and genipin were 5.73 and 6.65, respectively. The elution order was the same observed by other authors (Bentes & Mercadante, 2014; Wang et al., 2016). However, duration of the methods reported in the literature is much longer (up to 75 min) when compared to the obtained in this study (13 min). Resolution, width of peaks, selectivity, symmetry factor and K prime were calculated by Empower 3 software and were for geniposide 1.62, 15.37, 1.05, 0.90 and 1.30; for genipin were 1.74, 15.37, 1.05; 0.91 and 1.67, respectively. These results indicate the good chromatographic method developed for the separation of iridoids because the resolution for the both compounds was higher than 1.5 and the symmetry factor was 0.90. The parameters mentioned above were not found in the methods reported in the literature.

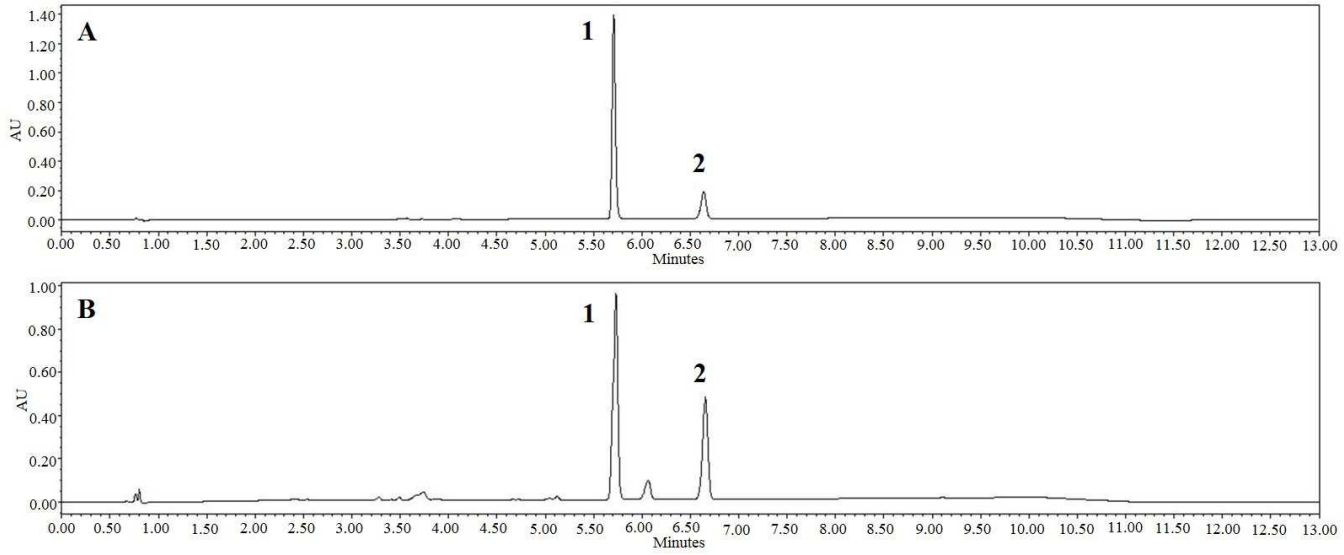

Figure 2. Representative chromatograms of the iridoids standards (A) and ethanolic extract of genipap (B). Geniposide (peak 1) and genipin (peak 2).

3.3 Method validation

Linearity and range

Linearity was determined for geniposide and genipin on eleven and ten levels of concentration, respectively. Geniposide showed a linear response from 0.41-1000 μg/mL and genipin showed a linear response from 0.41-625 μg/mL. All curves presented coefficients of linear correlation higher than 0.9998. Geniposide linearity found in the methods reported in the literature was between 1.0 and 1000 μg/mL (Bergonzi et al., 2012; Liu et al., 2011; Sheu & Hsin, 1998; Wu et al., 2014) and genipin linearity was between 0.5 and 100 μg/mL (Bentes & Mercadante, 2014; Wu et al., 2014).

Limit of detection and quantification

Geniposide and genipin at a concentration of 0.41 μg/mL presented a signal-to-noise ratio higher than 3:1, which were assumed as the limit of detection (LoD) for the both compounds. Geniposide at 6.5 μg/mL and genipin at 1.63 μg/mL presented a signal-to-noise ratio higher than 10:1, representing the limit of quantification (LoQ). The genipin limits were similar to reported by Bentes & Mercadante (2014), while the geniposide limits were approximately 10 times higher than the reported in the literature (Bergonzi et al., 2012; Liu et al., 2011; Wu et al., 2014).

Robustness

Table 1 shows the robustness results for the developed method concerning sample concentration/dilution and injection volume, respectively. The chromatographic performance was slightly affected by the sample concentration and injected volume. These results are related to the high performance of the fused-column used in this study because columns with this technology can operate with low amount of sample due to an increased diffusion of the sample in the solvent (Osorio-Tobón et al., 2016).

Precision and accuracy

The intraday and interday precision were evaluated in terms of retention time and peak area by injecting the sample 10 times within a day and by duplicating the experiment once a day during three consecutive days. The relative standard deviation (RSD) was lower than 0.04% for retention time and lower than 0.67% for peak area for the intraday precision. For interday precision the RSD was lower than 0.03% and 3.05% for retention time and peak area, respectively.

The accuracy of the developed method was determined by analyzing the percentage recovery of the both iridoids into different concentration levels of the genipap extract. As shown in Table 2, the geniposide recovery ranged between 95.0 and 96.8%, while the genipin recovery ranged between 103.5 and 110.5%.

Specificity

No deviations were observed in the geniposide and genipin UV-spectra at the beginning, at the apex and at the end of peaks of each constituent obtained from the genipap extract sample (Figure 3), demonstrating the purity of the peaks.

3.4 Application to real samples

To evaluate the performance of the chromatographic method, these iridoids were quantified in ethanolic extracts obtained from different parts of the genipap fruit. Figure 4 shows the chromatograms of the ethanolic extracts obtained for five different

Table 1. Effect of sample concentration and injection volume on the chromatographic performance.

	Compound	RT (min)	Concentration (μg/mL)	Width (s)	K prime	Selectivity	Resolution	Symmetry factor
Dilution								
$[X_0]/1$	Geniposide	5.730	351.56	15.57	1.30	1.06	1.56	0.96
	Genipin	6.649	125.01	15.20	1.67	1.05	-	0.92
$[X_0]/2$	Geniposide	5.734	337.53	15.37	1.30	1.05	1.62	0.90
	Genipin	6.653	121.15	15.37	1.67	1.05	1.74	0.91
$[X_0]/3$	Geniposide	5.733	344.72	15.10	1.30	1.04	2.12	0.87
	Genipin	6.651	125.40	14.77	1.67	1.05	-	0.91
$[X_0]/4$	Geniposide	5.658	306.67	15.03	1.30	1.05	1.70	0.87
	Genipin	6.597	111.80	15.70	1.67	1.07	2.65	0.90
$[X_0]/5$	Geniposide	5.729	360.40	14.97	1.30	1.06	1.69	0.85
	Genipin	6.648	132.52	15.70	1.67	1.07	3.92	0.91
Injection volume								
2.5	Geniposide	5.741	278.27	16.2	1.30	1.06	1.72	1.11
	Genipin	6.661	102.76	14.7	1.67	1.07	3.6	0.90
5	Geniposide	5.732	327.10	16.2	1.30	1.08	2.16	1.12
	Genipin	6.660	117.51	15.0	1.67	1.06	3.74	0.91
10	Geniposide	5.724	360.40	14.97	1.30	1.06	1.69	0.85
	Genipin	6.650	132.52	15.70	1.67	1.07	3.92	0.91
15	Geniposide	5.73	359.59	21.5	1.30	1.06	1.68	0.68
	Genipin	6.64	127.47	16.5	1.67	1.047	2.14	0.90
20	Geniposide	5.718	358.57	23.7	1.30	1.10	3.6	0.59
	Genipin	6.637	126.96	25.90	1.66	1.10	2.503	0.81

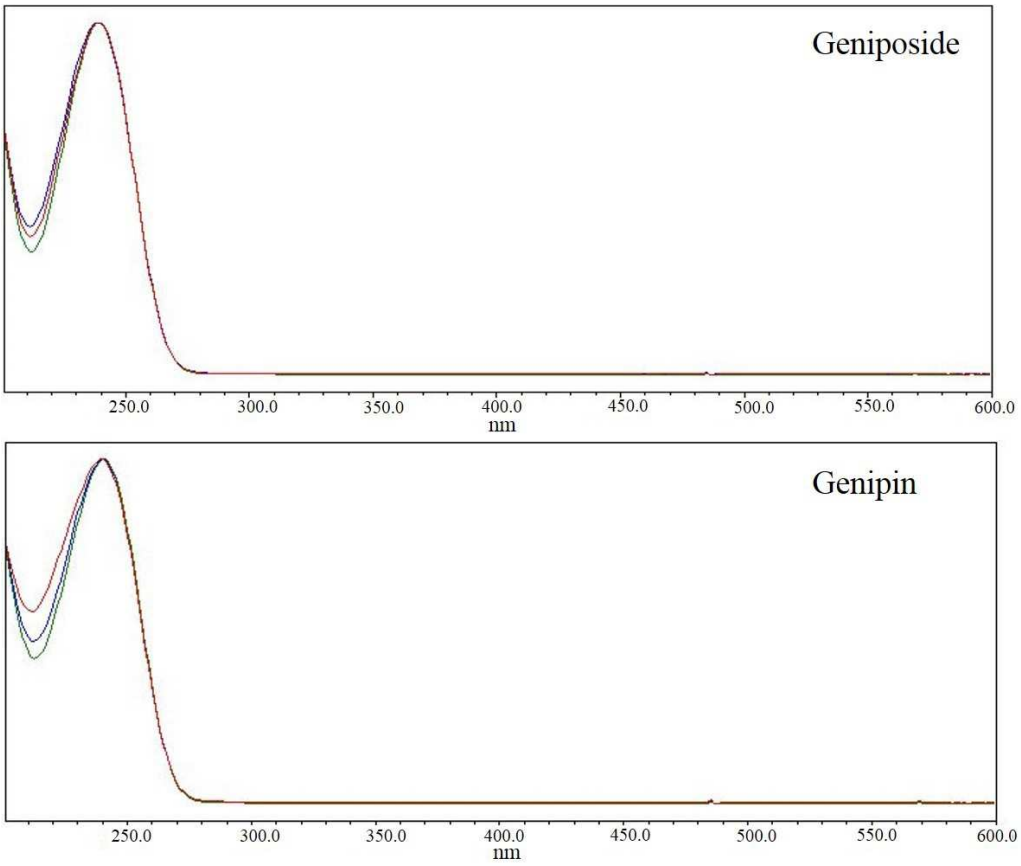

Figure 3. Overlay of three UV-spectra (240 nm) at the beginning, at the apex and at the end of the peaks of geniposide at 5.73 min and genipin at 6.65 min.

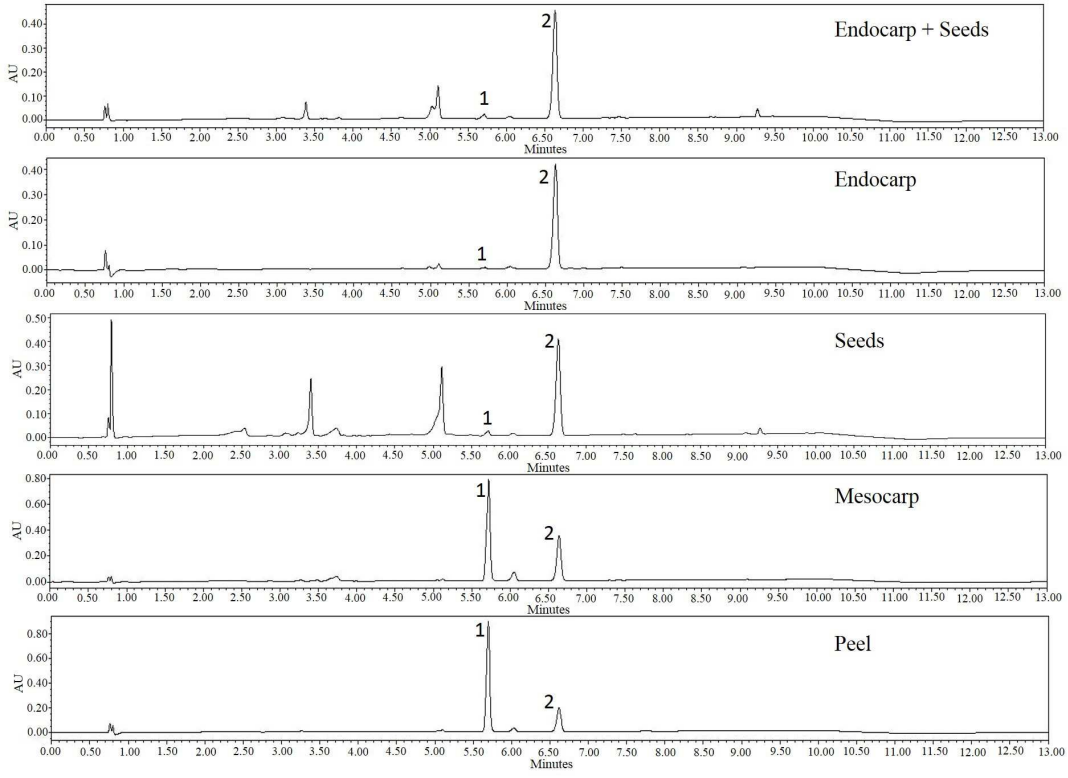

Figure 4. Representative chromatograms of the ethanolic extract from different parts of genipap fruit. Geniposide (peak 1) and genipin (peak 2).

Table 2. Recovery of iridoids.

Analyte	Original amount (μg)	Spiked (μg)	Found (μg)	Recovery (%)	RSD (%)
Geniposide	24.70	3.86	27.54	96.8	2.3
	12.84	3.86	16.12	96.4	0.3
	5.73	3.86	9.11	95.0	0.8
Genipin	8.64	3.13	12.15	103.5	2.1
	4.49	3.13	8.18	107.2	0.8
	2.01	3.13	5.71	110.5	1.1

RSD: Relative standard deviation.

Table 3. Concentration of iridoids (mg/g of raw material) in different unripe genipap fruit parts.

	Geniposide	Genipin	Total Iridoids
Endocarp + seeds	0.12	23.07	23.19
Endocarp	0.08	38.96	39.04
Seeds	0.06	1.17	1.23
Mesocarp	58.70	20.65	79.35
Peel	40.25	7.51	47.76

RSD: Relative standard deviation.

parts (endocarp + seeds, endocarp, seeds, mesocarp and peel) of the genipap fruit. The concentration of iridoids recovered from each part of the fruit is shown in Table 3. The iridoids profiles observed in Figure 4 were not directly proportional because the samples needed to be differently diluted in order to achieve the linear range for both compounds. Geniposide was mainly found in mesocarp (58.7 mg/g) and peel (40.25 mg/g) of the fruit, whereas significant amount of genipin was observed in all parts of the fruit except for seeds, which showed a content of 1.17 mg/g. Regarding the total iridoids recovery, the seeds also presented the lowest value (1.23 mg/g), while the mesocarp showed the highest (79.35 mg/g). This result corroborates the literature that reports the mesocarp as the main source of geniposide and the endocarp as the main source of genipin (Bentes & Mercadante, 2014). The results indicated that the proposed method was successfully applied to quantitatively analyze the main iridoids of the genipap fruit.

4 Conclusions

The two major iridoids from genipap fruit were accurately separated and quantified in a short time of 13 min of analysis. The method showed an excellent performance regarding the simplicity, precision, accuracy and robustness. The validated data showed a good performance for the different dilutions and injection volumes tested and also presented low deviation in terms of intermediate precision. This method has a great potential to be used by the industry for analysis of genipin and geniposide.

Acknowledgements

The authors are grateful to Sao Paulo Research Foundation (FAPESP 2015/13299-0) for financial support. G. Náthia-Neves thanks Coordination for the Improvement for the Higher Education Personnel (CAPES) for a Ph.D. assistantship. G.C. Nogueira and R. Vardanega thank National Council for Scientific and Technological Development (CNPq) for the P.D. assistantship (166060/2015-1 and 152148/2016-7). M.A.A. Meireles thanks CNPq for the productivity grant (302423/2015-0).

References

Bentes, A. S., & Mercadante, A. Z. (2014). Influence of the stage of ripeness on the composition of iridoids and phenolic compounds in genipap (*Genipa americana* L.). *Journal of Agricultural and Food Chemistry*, 62(44), 10800-10808. PMid:25323434. http://dx.doi.org/10.1021/jf503378k.

Bergonzi, M. C., Righeschi, C., Isacchi, B., & Bilia, A. R. (2012). Identification and quantification of constituents of *Gardenia jasminoides* Ellis (Zhizi) by HPLC-DAD-ESI-MS. *Food Chemistry*, 134(2), 1199-1204. PMid:23107748. http://dx.doi.org/10.1016/j.foodchem.2012.02.157.

Bianco, A. (1994). Recent developments in iridoids chemistry. *Pure and Applied Chemistry*, 66(10-11), 2335-2338. http://dx.doi.org/10.1351/pac199466102335.

Buchweitz, M. (2016). Natural solutions for blue colors in food. In R. Carle & R. Schweiggert (Eds.), *Handbook on natural pigments in food and beverages: industrial applications for improving food color* (355 p.). Cambridge: Elsevier.

Butler, M. F., Ng, Y.-F., & Pudney, P. D. A. (2003). Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. *Journal of Polymer Science. Part A, Polymer Chemistry*, 41(24), 3941-3953. http://dx.doi.org/10.1002/pola.10960.

Chisté, R. C., Yamashita, F., Gozzo, F. C., & Mercadante, A. Z. (2011). Simultaneous extraction and analysis by high performance liquid chromatography coupled to diode array and mass spectrometric detectors of bixin and phenolic compounds from annatto seeds. *Journal of Chromatography A*, 1218(1), 57-63. PMid:21111424. http://dx.doi.org/10.1016/j.chroma.2010.10.094.

Dinda, B., Debnath, S., & Harigaya, Y. (2007). Naturally occurring iridoids: a review, Part 1. *Chemical & Pharmaceutical Bulletin*, 55(2), 159-222. PMid:17268091. http://dx.doi.org/10.1248/cpb.55.159.

Djerassi, C., Gray, J. D., & Kincl, F. A. (1960). Naturally occurring oxygen heterocyclics. IX.[1] Isolation and characterization of genipin[2]. *The Journal of Organic Chemistry*, 25(12), 2174-2177. http://dx.doi.org/10.1021/jo01082a022.

Ferreira, M. K. L. (2015). Introduction. In M. K. L. Ferreira, *Mapping time, space and the body: indigenous knowledge and mathematical thinking in Brazil* (pp. 1-28). Rotterdam: SensePublishers.

International Council for Harmonisation – ICH. (2005). Harmonised tripartite guideline: validation of analytical procedures: text and methodology Q2 (R1). In *International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use* (pp. 13). Geneva: ICH.

Koo, H. J., Lim, K. H., Jung, H. J., & Park, E. H. (2006). Anti-inflammatory evaluation of gardenia extract, geniposide and genipin. *Journal of Ethnopharmacology*, 103(3), 496-500. PMid:16169698. http://dx.doi.org/10.1016/j.jep.2005.08.011.

Lee, E. J., Hong, J. K., & Whang, W. K. (2014). Simultaneous determination of bioactive marker compounds from Gardeniae fructus by high performance liquid chromatography. *Archives of Pharmacal Research*, 37(8), 992-1000. PMid:24277694. http://dx.doi.org/10.1007/s12272-013-0293-1.

Li, J., Xu, B., Zhang, Y., Dai, S., Sun, F., Shi, X., & Qiao, Y. (2016). Determination of geniposide in *Gardenia jasminoides* Ellis fruit by near-infrared spectroscopy and chemometrics. *Analytical Letters*, 49(13), 2063-2076. http://dx.doi.org/10.1080/00032719.2015.1130714.

Liu, H., Su, J., Liang, X., Zhang, X., He, Y.-J., Huang, H.-Q., Ye, J., & Zhang, W.-D. (2011). Identification and determination of the major constituents in traditional Chinese medicine Longdan Xiegan Pill by HPLC-DAD-ESI-MS. *Journal of Pharmaceutical Analysis*, 1(1), 1-7. http://dx.doi.org/10.1016/S2095-1779(11)70001-6.

Náthia-Neves, G., Tarone, A. G., Tosi, M. M., Maróstica Júnior, M. R., & Meireles, M. A. A. (2017). Extraction of bioactive compounds from genipap (*Genipa americana* L.) by pressurized ethanol: Iridoids, phenolic content and antioxidant activity. *Food Research International*, 102, 595-604. PMid:29195990. http://dx.doi.org/10.1016/j.foodres.2017.09.041.

Osorio-Tobón, J. F., Carvalho, P. I. N., Barbero, G. F., Nogueira, G. C., Rostagno, M. A., & Meireles, M. A. A. (2016). Fast analysis of curcuminoids from turmeric (*Curcuma longa* L.) by high-performance liquid chromatography using a fused-core column. *Food Chemistry*, 200, 167-174. PMid:26830575. http://dx.doi.org/10.1016/j.foodchem.2016.01.021.

Pino, J., Marbot, R., & Vazquez, C. (2005). Volatile constituents of genipap (*Genipa americana* L.) fruit from Cuba. *Flavour and Fragrance Journal*, 20(6), 583-586. http://dx.doi.org/10.1002/ffj.1491.

Prance, G. T. (2003). Fruits of tropical climates: fruits of Central and South America. In B. Caballero (Ed.), *Encyclopedia of food sciences and nutrition* (2nd ed., pp. 2810-2816). Oxford: Academic Press.

Ramos-de-la-Peña, A. M., Renard, C. M., Wicker, L., Montanez, J. C., Garcia-Cerda, L. A., & Contreras-Esquivel, J. C. (2014). Environmental friendly cold-mechanical/sonic enzymatic assisted extraction of genipin from genipap (Genipa americana). *Ultrasonics Sonochemistry*, 21(1), 43-49. PMid:23871416. http://dx.doi.org/10.1016/j.ultsonch.2013.06.008.

Ramos-de-la-Peña, A. M., Renard, C. M. G. C., Montañez, J. C., Reyes-Vega, M. L., & Contreras-Esquivel, J. C. (2015a). Ultrafiltration for genipin recovery technologies after ultrasonic treatment of genipap fruit. *Biocatalysis and Agricultural Biotechnology*, 4(1), 11-16. http://dx.doi.org/10.1016/j.bcab.2014.09.009.

Ramos-de-la-Peña, A. M., Montañez, J. C., Reyes-Vega, M. L., & Contreras-Esquivel, J. C. (2015b). Temperature model for process impact non-uniformity in genipin recovery by high pressure processing. *Food Chemistry*, 187, 444-450. PMid:25977049. http://dx.doi.org/10.1016/j.foodchem.2015.04.114.

Rostagno, M. A., Debien, I. C. N., Vardanega, R., Nogueira, G. C., Barbero, G. F., & Meireles, M. A. A. (2014). Fast analysis of beta-ecdysone in Brazilian ginseng (*Pfaffia glomerata*) extracts by high-performance liquid chromatography using a fused-core column. *Analytical Methods*, 6(8), 2452-2459. http://dx.doi.org/10.1039/C3AY42276C.

Sheu, S. J., & Hsin, W. C. (1998). Identification and determination of the major constituents in traditional Chinese medicine Longdan Xiegan Pill by HPLC-DAD-ESI-MS. *Journal of Separation Science*, 21(9), 523-526.

Thomas, P., & Farrugia, K. (2013). An investigation into the enhancement of fingermarks in blood on paper with genipin and lawsone. *Science & Justice*, 53(3), 315-320. PMid:23937940. http://dx.doi.org/10.1016/j.scijus.2013.04.006.

Velásquez, C. L., Rivas, A., & Ocanto, I. S. (2014). Obtención de Genipina a partir de frutos de caruto (*Genipa americana* L.) del llano venezolano. *Avances en Química*, 9(2), 75-86.

Wang, L., Liu, S., Zhang, X., Xing, J., Liu, Z., & Song, F. (2016). A strategy for identification and structural characterization of compounds from *Gardenia jasminoides* by integrating macroporous resin column chromatography and liquid chromatography-tandem mass spectrometry combined with ion-mobility spectrometry. *Journal of Chromatography. A*, 1452, 47-57. PMid:27208986. http://dx.doi.org/10.1016/j.chroma.2016.05.026.

Wu, X., Zhou, Y., Yin, F., Mao, C., Li, L., Cai, B., & Lu, T. (2014). Quality control and producing areas differentiation of Gardeniae Fructus for eight bioactive constituents by HPLC–DAD–ESI/MS. *Phytomedicine*, 21(4), 551-559. PMid:24183952. http://dx.doi.org/10.1016/j.phymed.2013.10.002.

Zabot, G. L., Moraes, M. N., Rostagno, M. A., & Meireles, M. A. A. (2014). Fast analysis of phenolic terpenes by high-performance liquid chromatography using a fused-core column. *Analytical Methods*, 6(18), 7457-7468. http://dx.doi.org/10.1039/C4AY01124D.

- *CHAPTER 4* -

EXTRACTION OF BIOACTIVE COMPOUNDS OF GENIPAP BY PRESSURIZED ETHANOL

Extraction of bioactive compounds from genipap (*Genipa americana* L.) by pressurized ethanol: iridoids, phenolic content and antioxidant activity

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Article published in the journal Food Research International, vol. 102., p. 595-604, 2017

ISSN: 0963-9969. DOI: 10.1016/j.foodres.2017.09.041

Received 12 June 2017, Revised 12 September 2017, Accepted 17 September 2017

Food Research International 102 (2017) 595–604

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Extraction of bioactive compounds from genipap (*Genipa americana* L.) by pressurized ethanol: Iridoids, phenolic content and antioxidant activity

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ARTICLE INFO

Keywords:
Genipin
Geniposide
Natural dyes
Green extraction
Genipa americana L

ABSTRACT

The search for compounds with functional properties from natural sources has grown in recent years as people have developed healthier habits. Therefore, the aim of this study was to evaluate the extraction of bioactive compounds from various parts of unripe genipap fruit (*Genipa americana* L.) by using pressurized ethanol to verify which part of the fruit provides the greatest recovery of the iridoids genipin and geniposide. Two process variables were studied: temperature (50 and 80 °C) and pressure (2, 12 and 20 bar). The whole fruit and the peel, mesocarp, endocarp, endocarp + seeds and seeds of the fruit were studied. The endocarp presented with the highest recovery of genipin (48.6 ± 0.6 mg/g raw material) and the extraction from the mesocarp allowed a greater recovery of geniposide (59 ± 1 mg/g raw material). The highest values of total phenolic content were obtained with mesocarp extracts. The endocarp and mesocarp extracts presented the highest antioxidant activity as measured by FRAP and DPPH. These results are promising and support the use of unripe genipap fruit as a source of iridoids and natural antioxidants.

1. Introduction

Genipap (*Genipa americana* L.) is a native fruit from Brazil that belongs to the *Rubiaceae* family (Oliveira, Yamada, Fagg, & Brandão, 2012). This fruit can also be found in Central and South America and is popularly consumed in juices, liqueurs and jellies.

Unripe genipap has been used since ancient times by indigenous people for body painting (Ferreira, 2015). The color of the unripe genipap fruit is due to an iridoid called genipin (Bentes & Mercadante, 2014). Genipin is a polar and colorless substance that reacts spontaneously with the primary amines of amino acids, peptides or proteins in the presence of oxygen to form blue pigments (Djerassi, Gray, & Kincl, 1960). Genipin can be extracted from genipap with organic solvents or by the enzymatic hydrolysis of geniposide with β-glycosidases (Ramos-de-la-Peña, Renard, Montañez, Reyes-Vega, & Contreras-Esquivel, 2015; Thomas & Farrugia, 2013). Geniposide is also an iridoid present in genipap fruits (Butler, Ng, & Pudney, 2003). This compound is the main iridoid glycoside found in ripe Gardenia fruit (*Gardenia jasminoides* Ellis), which also belongs to the *Rubiaceae* family. Geniposide is often used in Asian countries as a natural yellow dye (Xiao, Li, Wang, & Ho, 2017).

The ripeness of genipap is an important factor to observe when extracting iridoids because genipin and geniposide are present only in unripe genipap fruits (Bentes, de Souza, Amaya-Farfan, Lopes, & de Faria, 2015; Renhe, Stringheta, Silva, & Oliveira, 2009). According to Bentes and Mercadante (2014), the total iridoid content decreases by 90% during the ripening process. The main visual differences between ripe and unripe fruits are the firmness and color of the fruits (Bentes & Mercadante, 2014). Unripe fruit has a gray-colored, firm peel and green flesh while the ripe fruit has a dark brown-colored peel that is rough and wilted, and its pulp turns light brown in color.

In addition to iridoids, genipap is also a source of phenolic compounds with high antioxidant potential (Bentes & Mercadante, 2014; Omena et al., 2012). These bioactive compounds that are present in genipap have attracted the interest of the scientific community due to their beneficial effects on human health. For example, genipin can act as an antimicrobial, anti-inflammatory and anti-cancer agent (Kim et al., 2005; Koo et al., 2004; Koo, Lim, Jung, & Park, 2006); geniposide exhibits a protective effect in asthma (Deng et al., 2013) and may be a novel regulator of insulin signaling (Zhang et al., 2015); and the

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http://dx.doi.org/10.1016/j.foodres.2017.09.041
Received 12 June 2017; Received in revised form 12 September 2017; Accepted 17 September 2017
Available online 19 September 2017
0963-9969/

antioxidants protect biological systems against the formation of free radicals, which contribute to the onset of diseases such as cancer (Omena et al., 2012).

Despite these functional properties, the extraction of bioactive compounds from genipap is still poorly studied, especially in the unripe fruits. Genipap can be divided into the following parts: peel, mesocarp, endocarp and seeds. According to Bentes and Mercadante (2014), the endocarp presents the highest content of genipin while the mesocarp presents the highest geniposide content. However, the other parts of the fruit were not explored.

Extraction of the compounds present in genipap fruit can be performed by PLE (pressurized liquid extraction), which is an environmentally conscious technology that obtains high yields despite using significantly lower amounts of solvents than traditional technics. This technique has been improved by the use of GRAS (Generally Recognized As Safe) solvents, which give the final extract a greater purity and lower toxicity. In addition, this process is selective because it is possible to extract either polar or nonpolar compounds, depending on the characteristic of the solvents used (Vazquez-Roig & Picó, 2015).

Thus, the aim of this study was to evaluate genipin and geniposide extraction by PLE from the peel, mesocarp, endocarp, seeds, endocarp + seeds and the whole fruit of unripe genipap and to analyze the phenolic content and antioxidant activity of the obtained extracts.

2. Materials and methods

2.1. Chemicals

For the extractions, ethyl alcohol absolute with 99.0% purity was purchased from Dinâmica (Diadema, Brazil). The HPLC standards of genipin (purity > 98%) and geniposide (purity > 98%) were purchased from Sigma-Aldrich (St. Louis, USA). Acetonitrile was of chromatography grade and purchased from J. T. Baker (Phillipsburg, USA). Ultrapure water was supplied by a Milli-Q Advantage 8 Purifier System from Millipore (Bedford, USA). Formic acid was purchased from Dinâmica (Diadema, Brazil). For assays of total phenolic content, Folin–Ciocalteu reagent was purchased from Dinâmica (Diadema, Brazil), gallic acid was purchased from Sigma-Aldrich (St. Louis, USA) and sodium carbonate was purchased from Labsynth (Diadema, Brazil). For assaying antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich (St. Louis, USA). Glacial acetic acid, ferric chloride ($FeCl_3$), hydrogen chloride and sodium acetate were purchased from Labsynth (Diadema, Brazil).

2.2. Sample preparation

Unripe genipap fruits were acquired from Sítio do Bello (Paraibuna, Brazil) in February 2016. Qualitative tests of firmness (manual) and peel color (visual) were used as criteria to assure the unripe stage of the fruits. Genipap fruits were collected from different trees, washed and stored in a freezer at − 18 °C until analysis of the chemical composition and extractions. The fruits were randomly selected to perform the experiments and were separated into the following parts with a knife: whole fruit, peel, mesocarp, endocarp, seeds and endocarp + seeds. The endocarp + seeds and whole fruit were studied to determine if together these parts contain a high content of the bioactive compounds (iridoids, phenolic content and antioxidant activity), which would thus eliminate the cost and time required for separating the fruit into parts. After separation, each part was homogenized in a mixer. Fig. 1 shows the parts of the fruit that were used. The proportion of each part of the unripe genipap fruit is as follows: peel 12 ± 3%; seeds 11 ± 4%; mesocarp 57 ± 5%; endocarp 12 ± 3%; and endocarp + seeds 27 ± 5%.

2.3. Chemical composition

Moisture and ash were determined according to the AOAC (1997) by methods n° 920.151 and 923.03, respectively. The proteins were determined by method 970.22 using a conversion factor of 6.25 (AOAC, 1997). The lipid content was analyzed according to the method of Bligh and Dyer (1959). Total dietary fiber content was determined by method 985.29 from the AOAC (1990) and Prosky and Lee (1996). Carbohydrate content was calculated by formula: 100 − (% ash + % lipids + % protein + % total dietary fiber). All analyses were performed in triplicate.

2.4. Extraction procedure

The PLE process is mainly affected by the chosen solvent, temperature, and extraction time (directly related to the solvent to feed ratio) and, to a lesser extent, by the pressure applied (Osorio-Tobón & Meireles, 2013). In this study, ethanol was selected as the solvent because it is a GRAS (Generally Recognized As Safe) solvent widely used for the extraction of polar compounds and is more selective than water (Osorio-Tobón & Meireles, 2013). The temperature (50 and 80 °C) was selected according to reports in the literature (Renhe et al., 2009). The extraction time was defined by previous experiments, as well as the solvent to feed ratio. There are no studies reporting the use of different pressures for genipin extraction from genipap, so the pressures (2, 12 and 20 bar) were selected according to the experience of our research group (LASEFI).

PLE was performed in a homemade unit shown in Fig. 2. For each assay, approximately 3.5–6 g of raw material was placed in an extraction vessel (5 mL) (Waters, serial # 4501374824-10, Pittsburg, USA) that contained a sintered metal filter at its bottom and top. Wet samples of raw material were used to eliminate the costs related to drying steps. The extraction vessel was connected to the system and heated by an electrical heating jacket. Then, the extraction vessel was filled with ethanol by an HPLC pump (Thermoseparation Products, California, USA) until the desired pressure was reached, and the pressure was maintained for 5 min for the static extraction. Thereafter, the micrometer (Autoclave Engineers, 10VRMM2812, Erie, USA) and back-pressure (Tescom, 26-1761-24-161, ELK River, USA) valves were opened and carefully adjusted to maintain the system's pressure. The solvent to feed ratio (S/F) used was 5 (wet basis). The ethanol extract was collected in glass flasks submerged in ice and stored under freezing temperatures (− 18 °C) in the absence of light until further analyses. The ethanol was removed from the extracts with a rota-evaporator (Marconi, MA120, Piracicaba, Brazil) at 50 °C.

2.5. Extract evaluation

2.5.1. Global yield

The global yield of the extracts obtained by PLE was calculated as the ratio of the total mass extracted (M_{ext}) to the mass of the raw material used to feed the system in dry basis (F), according to the following Eq. (1):

$$\text{Global Yield (\%)} = \left(\frac{M_{ext}}{F}\right)^{*} 100 \quad (1)$$

2.5.2. Iridoids quantification

The extracts obtained by PLE were filtered through a 0.45 μm filter and then analyzed using the HPLC-PDA (Waters, Alliance E2695, Milford, USA) system, consisting of a separation module (2695) with an integrated column heater, autosampler and photodiode array (PDA) detector. The analysis was performed by a method developed and validated by Náthia-Neves in a previous study (data not published). Separation of the iridoids was carried out on a fused-core C18 column (Kinetex, 100 × 4.6 mm i.d.; 2.6 μm; Phenomenex, Torrance, USA)

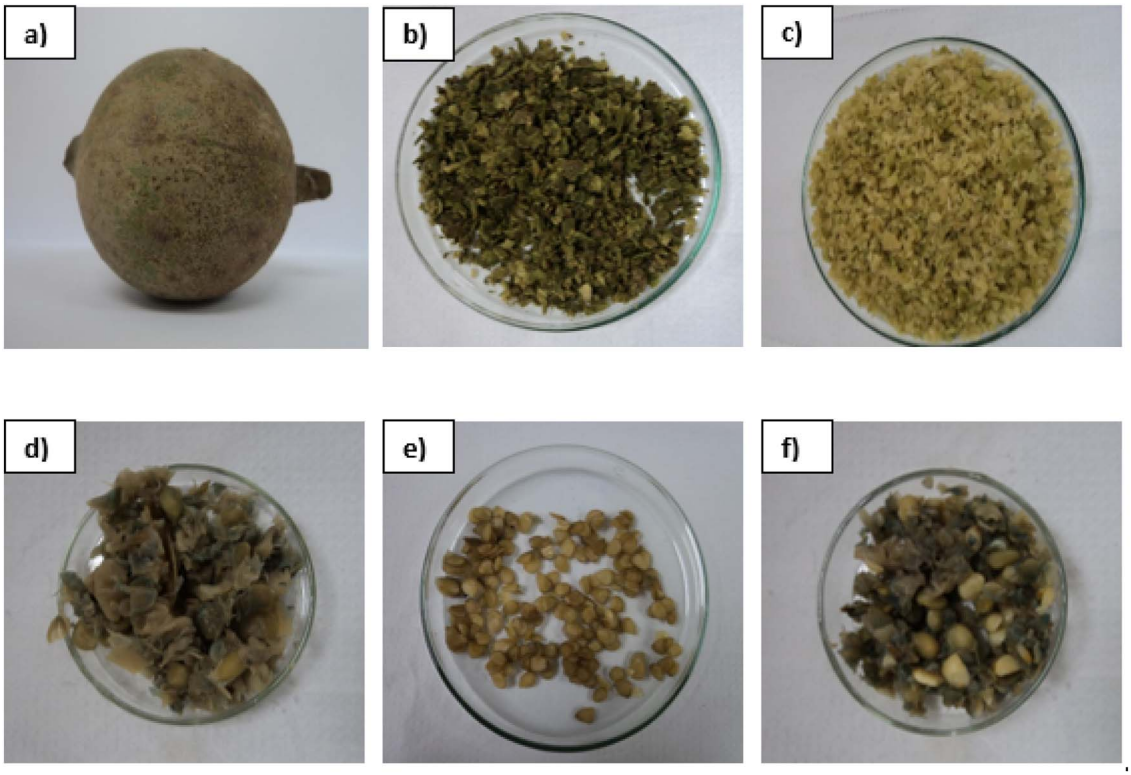

Fig. 1. Characteristics of the parts of the genipap fruit: a) Whole fruit; b) Peel; c) Mesocarp; d) Endocarp; e) Seeds; and f) Endocarp + seeds.

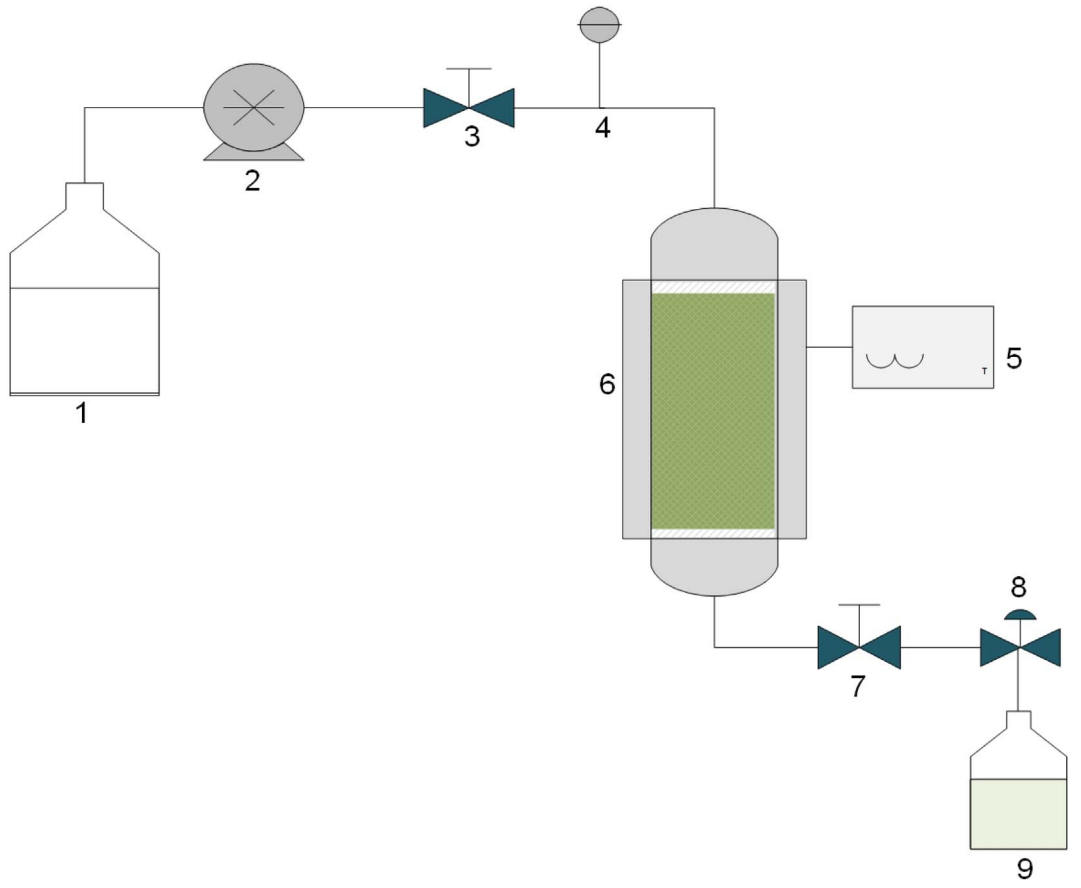

Fig. 2. Extraction equipment. (1) - Solvent reservoir; (2) - HPLC pump; (3) - Blocking Valve; (4) - Manometer; (5) - Temperature controller; (6) - Extraction vessel; (7) - Blocking Valve; (8) - Back pressure valve; (9) - Sampling bottle.

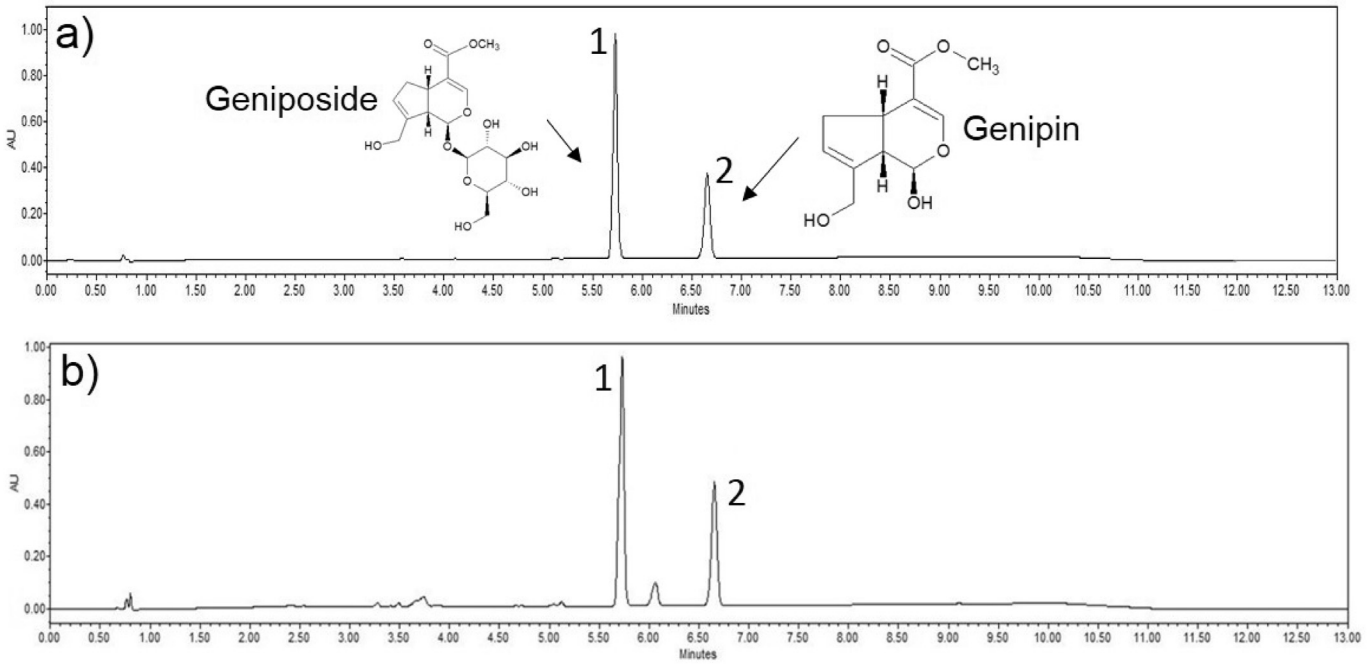

Fig. 3. Representative chromatograms of the iridoids: a) Standard solution of genipin (104 μg/mL) and geniposide (312 μg/mL); and b) Ethanol extract from mesocarp obtained at 50 °C and 2 bar. Geniposide (peak 1) and genipin (peak 2).

using a mobile phase of water (A) and acetonitrile (B) that were both acidified with 0.1% formic acid and the following gradient: 0 min, 99% A; 9 min, 75% A; 10 min, 99% A and 13 min, 99% A. The temperature and flow rate were 35 °C and 1.5 mL/min, respectively. The calibration curves of the iridoids were obtained at the range of 0.1–1000 μg mL^{-1} for geniposide (R^2 = 0.9998) and 0.1–625 μg mL^{-1} for genipin (R^2 = 0.9998). Fig. 3 shows the chromatograms of the standards and the mesocarp extracts.

2.5.3. Color analysis

The color was measured in a Hunterlab colorimeter (Hunter Associates Laboratory, Inc., Reston, VA, USA) equipped with a D65 light source and an angle of observation of 2° for all samples. The color was characterized with a CIELAB system, whose color coordinates L*, a* and b* indicate the intensity of the color as well as its chromatic perception. The parameter related to luminosity (L*) is the attribute related to the light transmission observed, indicating its intensity by its similarity with the color black (L* = 0) and the color white (L* = 100). The parameter a* indicates the likeness of the sample color to green (−) and red (+), while parameter b* indicates the proximity to the blue (−) and yellow (+) colors. With the parameters L*, a* and b*, the cylindrical coordinates C* (chroma) and H* (Hue angle) were calculated, which define the intensity and tone of the samples according to Eqs. (2) and (3), respectively. The extract color analysis was carried out at room temperature, and the sampling was performed in triplicate.

$$C^* = \sqrt{(a^{*2} + b^{*2})} \quad (2)$$

$$H^* = \arctan\left(\frac{b^*}{a^*}\right) \quad (3)$$

2.5.4. Total phenolic content (TPC) and antioxidant activity

TPC was determined using the Folin-Ciocalteau method as described by Singleton, Orthofer, and Lamuela-Raventós (1999). The absorbance was read at 725 nm. Gallic acid was used to plot the standard curve (16 to 120 mg/mL). All analyses were carried out in triplicate, and the results were expressed in mg of GAE (gallic acid equivalent) per g of dry raw material (mg GAE/g RM).

The antioxidant activity of the extracts obtained from each part of genipap fruit was determined by free radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP). The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed according to Brand-Williams, Cuvelier, and Berset (1995). The decrease in the absorbance of the samples and the Trolox standard curve (plotted at 2.5–400 μM TE) were read at 515 nm after a 30 min reaction. The results are expressed as μmol of Trolox Equivalent (TE) per g of dry raw material (μmol TE/g RM). The FRAP assay was performed according to Rufino et al. (2010). The FRAP reagent was prepared in the dark with 300 mmol L^{-1} acetate buffer (pH 3.6), 10 mmol L^{-1} TPTZ (2,4,6-tris (2-pyridyl)-S-triazine) in a 40 mmol L^{-1} HCl solution with 20 mmol L^{-1} $FeCl_3$. The samples and Trolox standard curve (2.5 to 400 μM TE) were read at 595 nm. The results are expressed as μmol of Trolox equivalent per g of dry raw material (μmol TE/g RM). Absorbance values were read in a microplate reader SynergyHT, Biotek (Winooski, USA) with Gen5™2.0 data analysis software spectrophotometer.

2.6. Statistical analysis

Analyses of the influence of the parameters on global yield, genipin, geniposide, total phenolic content and antioxidant activity were performed by analysis of variance (ANOVA) using the Minitab 16® software (Minitab Inc., State College, PA, USA) with a 95% confidence level (p-value ≤ 0.05). The parameters were evaluated with a randomized full factorial design (2 × 3 × 6) with temperature (50 and 80 °C), pressure (2, 12 and 20 bar), and parts of genipap (peel, mesocarp, endocarp, seeds, endocarp + seeds and whole fruit), resulting in 36 total experimental runs (Table 1).

3. Results and discussion

3.1. Characterization of genipap parts

Table 2 presents the chemical composition of the whole fruit, mesocarp, peel, endocarp, endocarp + seeds and seeds from the unripe genipap fruits. All fruit parts, except the seeds and the endocarp

Table 1
Summary of the process parameters and results of the extraction process of bioactive compounds by PLE (results expressed on dry basis).

Genipap parts	T (°C)	P (bar)	X0 (%) SD = 2.1	Genipin content (mg/g RM) SD = 4.2	Geniposide content (mg/g RM) SD = 1.7	TPC (mg GAE/g RM) SD = 0.9	FRAP (μmol TE/g RM) SD = 2.9	DPPH (μmol TE/g RM) SD = 0.6
Mesocarp	50	2	46.9 ± 0.1	20.7 ± 0.9	59 ± 1	5.2 ± 0.2	6.5 ± 0.3	6.7 ± 0.2
		12	40.4 ± 0.3	12.5 ± 0.9	46.9 ± 0.8	5.7 ± 0.2	6.1 ± 0.4	6.1 ± 0.2
		20	38.9 ± 0.2	15.3 ± 0.8	45.4 ± 0.7	5.5 ± 0.2	5.1 ± 0.5	6.3 ± 0.2
	80	2	46.5 ± 0.4	2.93 ± 0.08	47.6 ± 0.9	7.4 ± 0.2	13.9 ± 0.6	8.9 ± 0.3
		12	47.5 ± 0.2	2.14 ± 0.05	46.1 ± 0.7	8.6 ± 0.3	13.6 ± 0.5	8.9 ± 0.2
		20	44 ± 1	1.66 ± 0.03	41.9 ± 0.9	8.3 ± 0.3	12.6 ± 0.6	9 ± 0.3
Seeds	50	2	14 ± 1	1.16 ± 0.02	0.06 ± 0.00	2.3 ± 0.2	5.3 ± 0.2	2.5 ± 0.1
		12	17.3 ± 0.1	2.68 ± 0.07	0.12 ± 0.00	2.6 ± 0.1	5.4 ± 0.2	2.8 ± 0.1
		20	14.79 ± 0.03	1.45 ± 0.09	0.01 ± 0.00	2.8 ± 0.2	5.5 ± 0.3	2.2 ± 0.1
	80	2	17.7 ± 0.1	1.6 ± 0.2	0.08 ± 0.00	2.1 ± 0.1	7.7 ± 0.3	1.46 ± 0.01
		12	19 ± 1	0.50 ± 0.06	0.05 ± 0.00	3 ± 0.3	9.2 ± 0.4	3.4 ± 0.1
		20	14.8 ± 0.1	0.60 ± 0.08	0.04 ± 0.00	2.32 ± 0.03	6.3 ± 0.3	1.8 ± 0.1
Peel	50	2	22.20 ± 0.02	7.5 ± 0.4	39.9 ± 0.7	1.6 ± 0.1	5.2 ± 0.2	1.8 ± 0.1
		12	23.3 ± 0.1	7.3 ± 0.3	39.7 ± 0.9	2.1 ± 0.1	11.4 ± 0.6	2.65 ± 0.03
		20	19 ± 3	6.9 ± 0.8	35.8 ± 0.9	1.8 ± 0.1	8.3 ± 0.4	1.56 ± 0.04
	80	2	23.9 ± 0.4	6.18 ± 0.07	34.5 ± 0.9	2.38 ± 0.02	15.3 ± 0.1	3.4 ± 0.2
		12	24 ± 1	6.57 ± 0.04	34.5 ± 0.6	2.3 ± 0.1	15.4 ± 0.6	3.8 ± 0.1
		20	20.97 ± 0.02	5.6 ± 0.4	31.9 ± 0.8	2.11 ± 0.03	11.8 ± 0.3	3.2 ± 0.1
Whole fruit	50	2	34.1 ± 0.3	37.2 ± 0.9	0.57 ± 0.01	4.5 ± 0.1	5.4 ± 0.3	4.40 ± 0.04
		12	35.3 ± 0.3	43.4 ± 1.3	1.47 ± 0.05	4 ± 0.3	5.4 ± 0.3	4.0 ± 0.1
		20	36.2 ± 0.2	46.5 ± 1.5	2.50 ± 0.07	3.2 ± 0.2	8.1 ± 0.4	5.4 ± 0.1
	80	2	39.155 ± 0.001	29 ± 1	0.45 ± 0.00	5.6 ± 0.3	11.9 ± 0.6	7.3 ± 0.1
		12	37.4 ± 0.1	24 ± 1	0.09 ± 0.00	6 ± 0.1	11.9 ± 0.8	6.7 ± 0.2
		20	37.6 ± 0.2	14.8 ± 0.9	0.04 ± 0.00	6.2 ± 0.2	12.3 ± 0.5	6.9 ± 0.1
Endocarp + seeds	50	2	14 ± 1	22.9 ± 0.9	0.10 ± 0.00	1.80 ± 0.03	2.5 ± 0.2	1.19 ± 0.03
		12	13.2 ± 0.5	21.2 ± 1.2	0.37 ± 0.00	1.6 ± 0.1	4.6 ± 0.3	1.7 ± 0.1
		20	10.4 ± 0.2	17.7 ± 0.9	0.06 ± 0.00	1.08 ± 0.03	3.4 ± 0.2	1.06 ± 0.03
	80	2	17.79 ± 0.07	7.42 ± 0.07	0.34 ± 0.00	2.9 ± 0.1	4.9 ± 0.4	1.38 ± 0.04
		12	15.8 ± 0.1	14.8 ± 0.6	0.31 ± 0.00	1.80 ± 0.04	5.2 ± 0.3	2.7 ± 0.1
		20	18.17 ± 0.04	12.4 ± 0.6	0.27 ± 0.00	3.4 ± 0.1	4.9 ± 0.4	2.3 ± 0.1
Endocarp	50	2	24 ± 1	38.9 ± 0.4	0.01 ± 0.00	1.99 ± 0.03	12.9 ± 0.6	3.0 ± 0.1
		12	25.3 ± 0.3	47.9 ± 0.9	0.01 ± 0.00	2.2 ± 0.1	11.4 ± 0.4	2.7 ± 0.1
		20	23.9 ± 0.5	34.1 ± 0.8	0.01 ± 0.00	1.7 ± 0.1	14.6 ± 1	2.13 ± 0.03
	80	2	25.9 ± 0.1	39.4 ± 0.5	0.33 ± 0.00	2.5 ± 0.1	26.5 ± 1	9.5 ± 0.3
		12	31.7 ± 0.2	48.6 ± 0.6	0.14 ± 0.00	2.9 ± 0.1	23.4 ± 0.6	11.4 ± 0.3
		20	22.2 ± 0.2	35.3 ± 0.3	0.17 ± 0.00	1.9 ± 0.1	11.6 ± 0.5	7.2 ± 0.2

X0: Global yield; TPC: Total phenolic content; RM: Raw material; GAE: Gallic acid equivalent; TE: Trolox equivalent; SD: Standard deviation by ANOVA (α = 0.05).

+ seeds, showed a moisture content above 75%, which is similar to other data reported in the literature. The mesocarp presented with the highest content of ash and carbohydrates, while the peel had the highest fiber content. Seeds were shown to be a good source of lipids and proteins, followed by the endocarp + seeds and the endocarp. Bentes et al. (2015) found that endocarp + seeds are 68% moisture, 2.75% ash, 9.97% proteins, 1.69% lipids, 46.05% total fiber and 39.54% carbohydrates on a dry basis. The same authors found that the mesocarp is 80.9% moisture, 4.97% ash, 3.24% proteins, 1.52% lipids, 41.19% total fiber and 49% carbohydrates on a dry basis. According to Figueiredo, Maia, Holanda, and Monteiro (1986), the endocarp is 74.67% moisture, 4.03% ash, 2.92% protein, 1.07% lipids, 7.11% total fiber and 84.87% carbohydrates on a dry basis. The seeds analyzed by Porto et al. (2014) presented with 69.2% moisture, 10.06% ash, 3.93% protein, 11.36% lipids and 74.64% carbohydrates on a dry basis. Data for the peel and whole fruit were not found in the literature, further highlighting the importance of this work. Minor differences between the data obtained in this work with those mentioned in the literature are expected because the chemical composition of the fruits can be influenced by several factors, such as harvesting time, maturation stage, variety, climate and soil conditions, sun exposure and post-harvest management (Souza, Pereira, Queiroz, Borges, & Carneiro, 2012).

3.2. Effect of the process parameters on global yield

Analysis of variance (ANOVA, α = 0.05) showed that the part of the fruit (p-value < 0.001), temperature (p-value = 0.003) and pressure (p-value = 0.044) significantly influenced global yield. Fig. 4 shows the mean values of global yield.

Mesocarp presented with the highest global yield, followed by whole fruit, endocarp, peel, seeds and endocarp + seeds (Fig. 4a). In comparison to the other fruit parts, mesocarp showed the highest

Table 2
Chemical composition of each part from genipap fruit.

	Moisture (%)	Ash (%, db)	Protein (%, db)	Lipids (%, db)	Total dietary fiber (%, db)	Carbohydrates (%, db)
Whole fruit	80.9 ± 0.6	4.94 ± 0.05	6.6 ± 0.3	3 ± 1	51 ± 3	33.7
Mesocarp	82.9 ± 0.5	6.18 ± 0.02	3.3 ± 0.2	4 ± 1	50 ± 8	37.0
Peel	75.58 ± 0.06	4.97 ± 0.08	4.4 ± 0.2	3.69 ± 0.08	64 ± 3	22.8
Endocarp + seeds	69.5 ± 3.5	3.84 ± 0.08	10.1 ± 0.2	5.6 ± 0.6	52 ± 6	28.6
Endocarp	78.4 ± 0.3	4.52 ± 0.07	7.7 ± 0.2	5.2 ± 0.3	50 ± 3	32.8
Seeds	52 ± 1	3.05 ± 0.05	13.6 ± 0.3	7.6 ± 0.6	52 ± 2	23.5

db: dry basis.

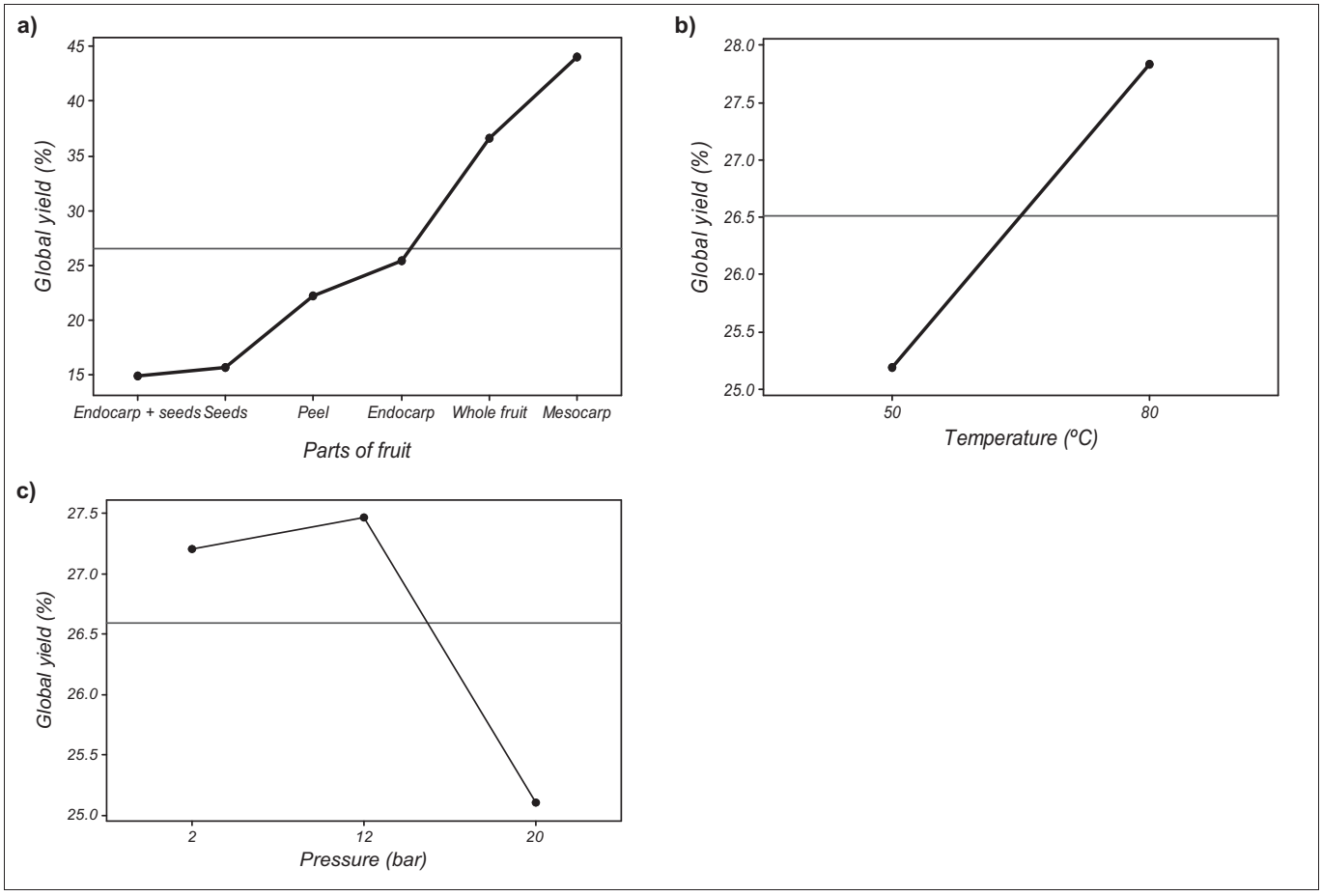

Fig. 4. Effect of the process parameters on global yield: a) Parts of fruit; b) Temperature; and c) Pressure.

moisture content, carbohydrates and ash, followed by whole fruit and endocarp. Carbohydrates can be extracted by water (Ruiz-Aceituno, García-Sarrió, Alonso-Rodriguez, Ramos, & Sanz, 2016) or hydroalcoholic mixtures (Buranov, Ross, & Mazza, 2010); thus, the water present in the fruit may have interacted with the ethanol used as the extracting solvent to form a hydroalcoholic mixture favoring the extraction of these compounds and consequently increasing the extraction yield of these parts.

The seeds and endocarp + seeds presented with a high lipid content (7.6 ± 0.6% and 5.6 ± 0.6, respectively), which is not easily extracted with ethanol due to the polarity of this solvent; therefore, the presence of these compounds in the vegetable matrix may have hampered the extraction of other compounds, resulting in lower global yields.

Fig. 4b shows that the increase in temperature increased global yield. This occurs because high temperatures break the Van der Waals, hydrogen and dipole-dipole molecular bonds between the extractable compounds and the vegetable matrix, reducing the required activation energy for their desorption. Furthermore, the viscosity and surface tension of the solvents decrease at higher temperatures, which favors the penetration of the solvent into the vegetable matrix, accelerating the mass transfer rate and leading to an increased extraction efficiency (Kamali, Khodaverdi, Hadizadeh, & Ghaziaskar, 2016; Machado, Pasquel-Reátegui, Barbero, & Martínez, 2015).

The influence of pressure on global yield is shown in Fig. 4c. The increase in pressure from 2 to 12 bar provided a slight increase in global yield; however, a steep drop in yield was observed when the pressure was increased from 12 to 20 bar. According to Osorio-Tobón, Carvalho, Rostagno, Petenate, and Meireles (2014) the increase in pressure may negatively interfere with the yield because it may promote changes in the raw material, reducing the surface contacts between the solvent and vegetable matrix. In addition, increasing the pressure may lead to a compaction of the raw material in the extraction bed, forming preferred paths that prevent proper contact between the solvent and the compounds to be extracted. It is also worth mentioning that the raw materials used in the extractions were wet, and this high-water content may have contributed to increasing bed compaction, reducing the extraction efficiency at high pressures.

Omena et al. (2012) studied extractions from the peel and seeds of ripe genipap fruit. In their study, the raw materials used were oven-dried at 35–40 °C, crushed and then extracted three times with 95% ethanol. The yields obtained by these authors were 36% for the peel and 25% for the seeds, but in the present study, the global yield for the peel ranged from 19 to 24% and from 14 to 19% for the seeds. These observed differences may be due to different extraction conditions (temperature, pressure, solvent, S/F, etc.), natural variations in the raw material, stage of ripeness, the effect of pretreatment or the use of wet raw material.

3.3. Effect of the process parameters on iridoid content

Fruit parts and temperature both had a significant interaction effect on the content of genipin (p-value = 0.010) and geniposide (p-value = 0.053), while the interaction between fruit parts and pressure only significantly affected the geniposide content (p-value = 0.050). Fig. 5 shows the mean values of the genipin and geniposide content obtained from each part of the genipap fruit.

According to Fig. 5a, the endocarp and whole fruit presented with the highest genipin content, followed by the endocarp + seeds, mesocarp, peel and seeds. The increased temperature had a negative effect

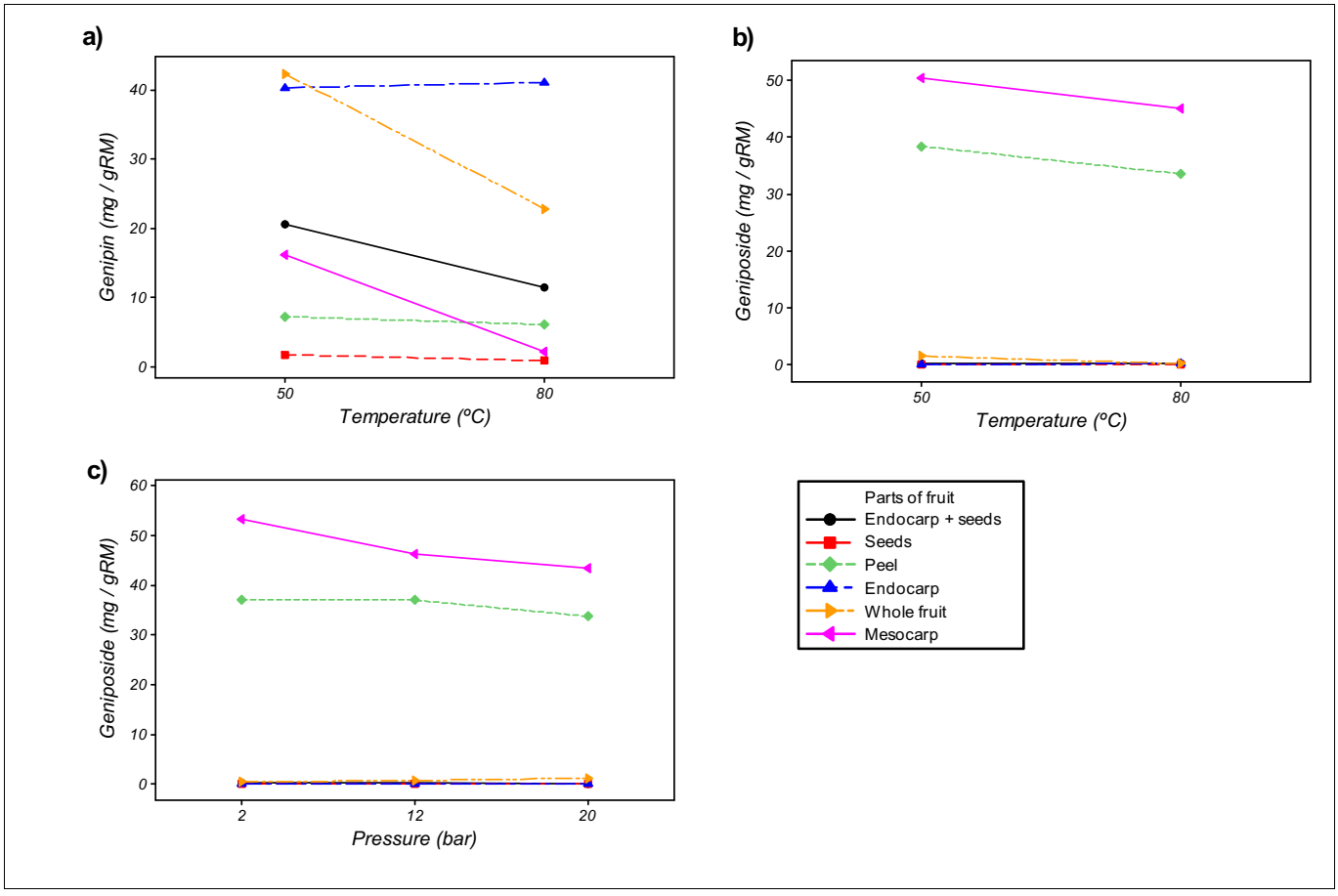

Fig. 5. Effect of the process parameters on iridoid content: a) Effect of the interaction between temperature and parts of the fruit on genipin content; b) Effect of the interaction between temperature and parts of the fruit on the geniposide content; and c) Effect of the interaction between pressure and parts of the fruit on the geniposide content. RM: Raw material.

on the genipin content with almost all fruit parts analyzed (except endocarp). An increase in temperature also had a negative effect on geniposide content (Fig. 5b). This result may be due to the rapid degradation of surface-solubilized iridoids, which decreased the recovery of these compounds at the higher temperature.

The best PLE condition for genipin recovery was with the endocarp at 80 °C and 12 bar (48.6 ± 0.6 mg/g RM, raw material), whereas the best condition for geniposide recovery was with the mesocarp at 50 °C and 2 bar (59 ± 1 mg/g RM); these results are in agreement with the results reported by Bentes et al. (2015), who studied the extraction of endocarp and mesocarp from unripe genipap fruits with a solution of methanol/water [8:2 (v/v)] by vortexing for 5 min at room temperature (22 ± 3 °C). These authors stated that endocarp contains the highest amount of genipin (3.4 ± 0.1 mg/g freeze dried sample) and mesocarp contains the highest amount of geniposide (118 ± 1 mg/g freeze dried sample). Ramos-de-la-Peña, Montañez, Reyes-Vega, Hendrickx, and Contreras-Esquivel (2015) also quantified genipin extraction from genipap fruit without seeds and recovered 34.0 ± 1.5 mg of genipin/g of the genipap fruit (wet basis) at 130 MPa using distilled water as a solvent.

Therefore, PLE from the wet endocarp extracted 14 times more genipin than that reported by Bentes et al. (2015). However, these authors extracted almost 2 times more geniposide than that reported in the present study. The geniposide, after hydrolysis, liberates the aglycone genipin (Lee, Lim, Bhoo, Paik, & Hahn, 2003). Thus, the temperatures employed in this study (50 and 80 °C) may have promoted the hydrolysis of geniposide and consequently resulted in larger amounts of genipin and lower amounts of geniposide.

Although the endocarp is the part of the fruit with the highest content of genipin, this part represents only 12 ± 3% of the fruit, which means that the recovery of genipin is 5.8 mg/g of fruit. By using the whole fruit, the genipin recovery was 46 mg/g of fruit, resulting in a recovery 8 times greater than with the endocarp. Thus, in a process optimization approach, it is important to consider not only the target compound content in the extract but also the process efficiency to recover as much of the compound from the vegetable matrix as possible.

3.4. Color

The parameters that define the color of the extracts obtained from each part of the fruit are presented in Table 3. The endocarp and endocarp + seeds presented with color in the blue region (b* negative). These results confirm the reports in the literature that the endocarp and endocarp + seed parts are used as a source to obtain a natural blue dye (Bentes et al., 2015; Brauch, Zapata-Porras, Buchweitz, Aschoff, & Carle, 2016). The extracts obtained at 80 °C from all parts, except the endocarp, presented with negative values for a*, placing these extracts in the green color region. The mesocarp, seeds, peel and whole fruit showed coloration in the yellow region (b* positive). Mesocarp, rich in geniposide, is used by Asian countries to obtain a natural yellow dye (Zhou et al., 2016). Although the extract obtained from the whole fruit contained a high amount of genipin, it presented with a green/yellow coloring. Thus, a purification process is necessary to obtain a natural dye with blue color from this extract.

3.5. Effect of the process parameters on TPC and antioxidant activity

Table 1 shows the TPC of the extracts obtained from each part of the

Table 3
Color parameters of each part extracted by genipap fruit.

	Parts of genipap	T (°C)	P (bar)	L*	a*	b*	C*	H
	Mesocarp	50	2	8.34 ± 0.02	−1.35 ± 0.2	2.86 ± 0.06	3.2 ± 0.1	115 ± 3
			12	9.76 ± 0.06	−1.88 ± 0.09	3.95 ± 0.08	4.37 ± 0.06	115 ± 1
			20	9.73 ± 0.04	−1.88 ± 0.2	3.54 ± 0.01	4 ± 0.1	118 ± 3
		80	2	5.91 ± 0.05	−1.27 ± 0.05	3.29 ± 0.04	3.59 ± 0.04	109.3 ± 0.9
			12	6.9 ± 0.1	−0.69 ± 0.09	2.2 ± 0.1	2.3 ± 0.1	106 ± 2
			20	7.45 ± 0.05	−1.28 ± 0.07	2.87 ± 0.04	3.1 ± 0.05	114 ± 1
	Seeds	50	2	8.74 ± 0.04	−0.83 ± 0.05	2.9 ± 0.1	3.1 ± 0.1	105.7 ± 0.3
			12	6.86 ± 0.08	−0.77 ± 0.06	1.4 ± 0.1	1.6 ± 0.09	119 ± 4
			20	10 ± 0.05	−0.57 ± 0.1	3.4 ± 0.1	3.49 ± 0.09	99.3 ± 2
		80	2	3.64 ± 0.02	−0.12 ± 0.03	−1.3 ± 0.2	1.3 ± 0.2	264 ± 2
			12	9 ± 0.1	−0.1 ± 0.2	2.7 ± 0.2	2.7 ± 0.2	92 ± 4
			20	3.79 ± 0.03	0.44 ± 0.2	−0.96 ± 0.04	1.07 ± 0.09	294 ± 9
	Peel	50	2	6.63 ± 0.08	−0.55 ± 0.2	7 ± 3	7.07 ± 3.07	95 ± 2
			12	6.3 ± 0.2	−0.22 ± 0.05	6.6 ± 0.3	6.6 ± 0.3	91.9 ± 0.5
			20	7.92 ± 0.09	−0.69 ± 0.04	6.58 ± 0.05	6.6 ± 0.05	96 ± 0.4
		80	2	8.8 ± 0.2	−1.53 ± 0.1	6.7 ± 0.2	6.9 ± 0.2	102.8 ± 0.9
			12	8.2 ± 0.1	−1.14 ± 0.06	6.8 ± 0.1	6.9 ± 0.1	99.6 ± 0.4
			20	8.42 ± 0.02	−1.05 ± 0.1	5.67 ± 0.05	5.77 ± 0.06	100.5 ± 0.9
	Whole fruit	50	2	10 ± 2	−2.01 ± 0.6	4.6 ± 2.4	5 ± 2	113.4 ± 9
			12	8.55 ± 0.03	−2.01 ± 0.07	5.6 ± 0.06	5.95 ± 0.07	109.8 ± 0.5
			20	8.58 ± 0.03	−2.21 ± 0.1	6.2 ± 0.1	6.5 ± 0.1	109.7 ± 0.6
		80	2	7.5 ± 0.1	−0.95 ± 0.03	1.94 ± 0.03	2.16 ± 0.03	116.1 ± 0.4
			12	4.79 ± 0.05	−0.6 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	108 ± 4
			20	5.04 ± 0.05	−0.58 ± 0.05	2.58 ± 0.04	2.65 ± 0.03	102.6 ± 0.9
	Endocarp + seeds	50	2	7 ± 2	−1.43 ± 0.7	−0.9 ± 0.6	1.7 ± 0.9	213 ± 3
			12	8.54 ± 0.02	−0.66 ± 0.04	−1.16 ± 0.02	1.3 ± 0.03	240 ± 1
			20	6.11 ± 0.04	−1.04 ± 0.02	−2.17 ± 0.07	2.4 ± 0.05	244 ± 1
		80	2	3.65 ± 0.05	−0.57 ± 0.08	−0.3 ± 0.07	0.58 ± 0.08	180 ± 6
			12	2.71 ± 0.02	−0.17 ± 0.05	−0.68 ± 0.4	0.07 ± 0.03	255 ± 5
			20	3.23 ± 0.06	−0.44 ± 0.05	−0.6 ± 0.1	0.07 ± 0.1	232 ± 3
	Endocarp	50	2	5.35 ± 0.07	−1.2 ± 0.1	−2.9 ± 0.2	3.2 ± 0.2	247.2 ± 0.8
			12	4.1 ± 0.1	−1.2 ± 0.2	−2.8 ± 0.2	3.1 ± 0.3	247 ± 2
			20	4.6 ± 0.3	−1.3 ± 0.2	−2.5 ± 0.2	2.8 ± 0.2	243 ± 4
		80	2	2.53 ± 0.09	0.6 ± 0.1	−2.2 ± 0.1	2.2 ± 0.1	285 ± 3
			12	1.43 ± 0.03	1.18 ± 0.06	−0.9 ± 0.1	1.5 ± 0.1	324 ± 3
			20	2.1 ± 0.06	0.5 ± 0.1	−1.56 ± 0.05	1.64 ± 0.07	288 ± 4

L*: luminosity: black (L* = 0) and White (L* = 100); a*: green color (−) and red color (+); b*: blue color (−) and yellow color (+); C*: croma; H*: angle hue. The images represent the extracts obtained at the 50 °C and 2 bar.

genipap fruit. Statistical analysis (ANOVA, α = 0.05) showed that the part of the fruit (p-value = 0.001) and the temperature (p-value < 0.001) had a significant effect on the TPC. Mesocarp had the highest TPC followed by whole fruit, seeds, endocarp and peel. The endocarp + seeds showed the lowest TPC (Fig. 6a).

There was an increase in TPC in all fruit parts studied when the temperature increased from 50 °C to 80 °C (Table 1). The positive effect of temperature increase on TPC has also been recorded by several other authors. Viganó et al. (2016) observed an increase from 23.9 ± 0.6 to 53 ± 1 mg GAE/g of bagasse in the TPC extraction of passion fruit bagasse with water and ethanol [50:50 (v/v)] when the temperature was increased from 50 °C to 70 °C. Garcia-Mendoza et al. (2017) observed that by increasing the temperature from 40 °C to 80 °C the TPC extraction from jussara residue with ethanol increased almost 62%. These results indicate that elevated temperatures have a positive effect on the solubility of phenolic compounds, which increases the mass transfer rate of these compounds into the solvent, improving the TPC extraction efficiency.

The antioxidant activity was measured by DPPH and FRAP methods and the results are shown in Table 1. The first method measures the ability of a given antioxidant compound to sequester free radicals or donate a hydrogen (Nithya & Madhavi, 2017), and the second method measures the antioxidant's ability to reduce ferric iron (Fe^{3+}) (Alam, Bristi, & Rafiquzzaman, 2013). The interaction between the fruit parts and temperature had a significant effect (p-value = 0.001) on the antioxidant activity measured by the DPPH method (Fig. 6b), but the parts of the fruit (p-value = 0.001) and temperature (p-value < 0.001) had a significant effect on the antioxidant activity measured by the FRAP method (Fig. 6c and Fig. 6d).

Mesocarp presented with the highest antioxidant activity by DPPH, and endocarp presented with the highest antioxidant activity by FRAP (Fig. 6b and c, respectively). As in TPC extractions, the temperature had a positive influence on the antioxidant activity.

There are no reports about the TPC and antioxidant activity of ethanol extracts obtained from unripe genipap fruits. The ripe pulp of genipap that was studied by Souza et al. (2012) presented with 48 ± 2 mg GAE/100 g pulp. This value is below the lowest value found in the present study for the endocarp (1.7 ± 0.1 mg GAE/g RM). Thus, the use of the unripe fruit can be advantageous for obtaining both iridoids and phenolic compounds with antioxidant activity.

4. Conclusion

The endocarp and whole fruit presented with the highest content of genipin while the mesocarp and peel presented with the highest content of geniposide. The temperature had a positive effect on global yield, total phenolic content and antioxidant activity. The mesocarp presented with a higher TPC and higher DPPH values, and the endocarp presented

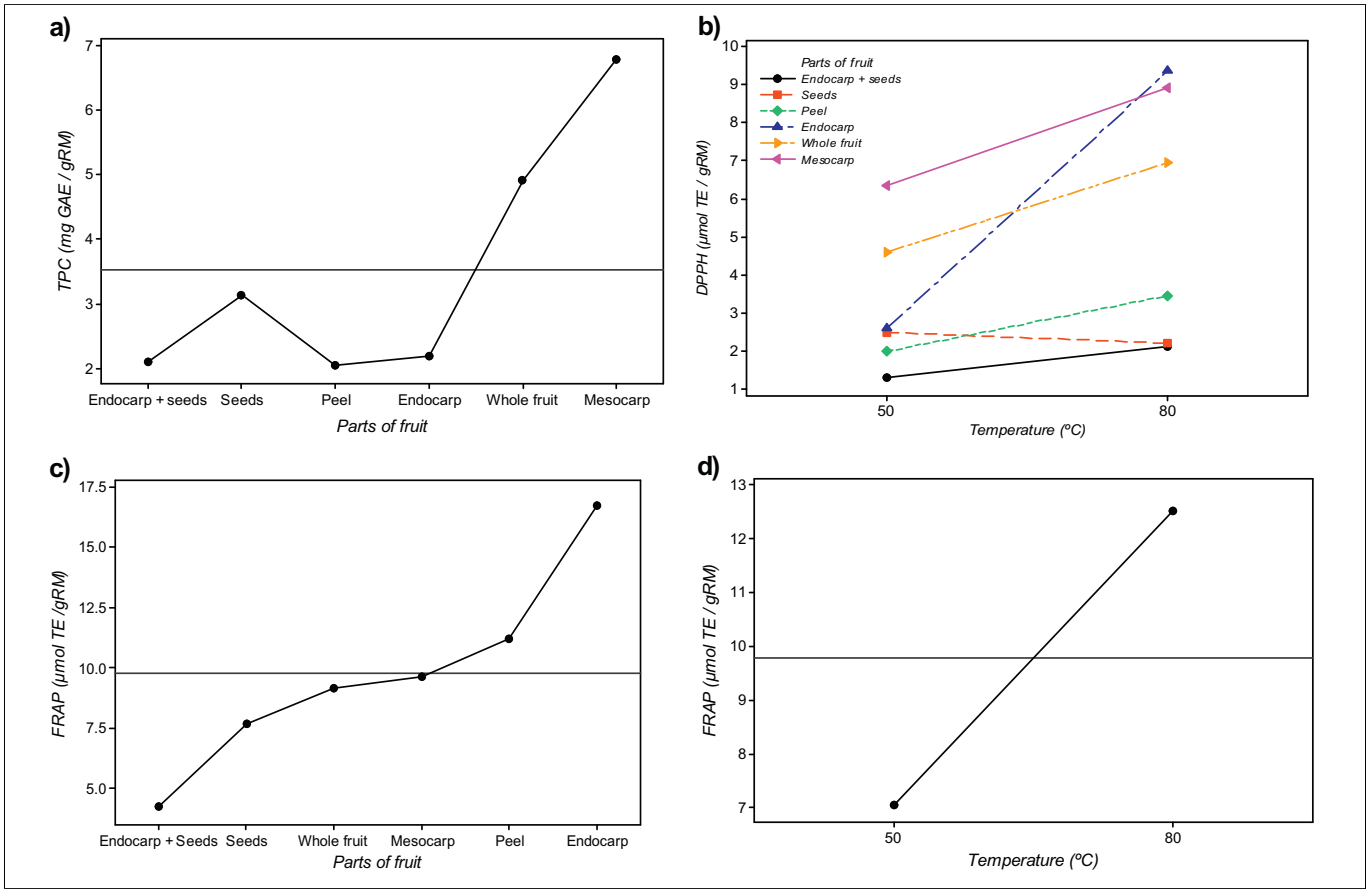

Fig. 6. Effect of the process parameters on TPC and antioxidant activity: a) Effect of parts of the fruit on TPC; b) Effect of the interaction between temperature and parts of the fruit on DPPH; c) Effect of the different fruit parts on FRAP; and d) Effect of temperature on FRAP. RM: Raw material.

with higher FRAP values. Thus, depending on the compound of interest, it is possible to use different parts of the genipap for extraction. Despite the high amount of genipin obtained from the whole plant, its use to recover the blue dye is conditioned by the necessity of further purification steps.

Acknowledgments

G. Náthia-Neves thanks CAPES/DEA/PROEX for a Ph.D. assistantship and M.A.A. Meireles thanks CNPq for the productivity grant (302423/2015-0). M.R. Marostica-Junior thanks CNPq for financial support (301108/2016-1).

References

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal, 21*(2), 143–152. http://dx.doi.org/10.1016/j.jsps.2012.05.002.

AOAC (1990). Official methods of analysis of the Association of Official Analytical Chemistry. Method 985.29. *Total dietary fiber in foods. Enzymatic-gravimetric method* (15th ed.). Arlington, VA: The association (1990).

AOAC (1997). *Official methods of analysis of the Association of Official Analytical Chemistry* (16th ed.). Gaithersburg, USA: AOAC International (1997).

Bentes, A. S., de Souza, H. A. L., Amaya-Farfan, J., Lopes, A. S., & de Faria, L. J. G. (2015). Influence of the composition of unripe genipap (*Genipa americana* L.) fruit on the formation of blue pigment. *Journal of Food Science and Technology, 52*(6), 3919–3924. http://dx.doi.org/10.1007/s13197-014-1651-9.

Bentes, A. S., & Mercadante, A. Z. (2014). Influence of the stage of ripeness on the composition of Iridoids and phenolic compounds in genipap (*Genipa americana* L.). *Journal of Agricultural and Food Chemistry, 62*(44), 10800–10808. http://dx.doi.org/10.1021/jf503378k.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*(8), 911–917.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology, 28*(1), 25–30. http://dx.doi.org/10.1016/S0023-6438(95)80008-5.

Brauch, J. E., Zapata-Porras, S. P., Buchweitz, M., Aschoff, J. K., & Carle, R. (2016). Jagua blue derived from *Genipa americana* L. fruit: a natural alternative to commonly used blue food colorants. *Food Research International*. http://dx.doi.org/10.1016/j.foodres.2016.08.029.

Buranov, A. U., Ross, K. A., & Mazza, G. (2010). Isolation and characterization of lignins extracted from flax shives using pressurized aqueous ethanol. *Bioresource Technology, 101*(19), 7446–7455. http://dx.doi.org/10.1016/j.biortech.2010.04.086.

Butler, M. F., Ng, Y.-F., & Pudney, P. D. A. (2003). Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. *Journal of Polymer Science Part A: Polymer Chemistry, 41*(24), 3941–3953. http://dx.doi.org/10.1002/pola.10960.

Deng, Y., Guan, M., Xie, X., Yang, X., Xiang, H., Li, H., ... Deng, X. (2013). Geniposide inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma. *International Immunopharmacology, 17*(3), 561–567. http://dx.doi.org/10.1016/j.intimp.2013.06.028.

Djerassi, C., Gray, J. D., & Kincl, F. A. (1960). Naturally occurring oxygen heterocyclics. IX.1 isolation and characterization of genipin2. *The Journal of Organic Chemistry, 25*(12), 2174–2177. http://dx.doi.org/10.1021/jo01082a022.

Ferreira, M. K. L. (2015). *Introduction mapping time, space and the body: Indigenous knowledge and mathematical thinking in Brazil.* Rotterdam: SensePublishers1–28.

Figueiredo, R. W., Maia, G. A., Holanda, L. F. F., & Monteiro, J. C. (1986). Características físicas e químicas do jenipapo. *Pesquisa Agropecuária Brasileira, 21*(4).

Garcia-Mendoza, M.d. P., Espinosa-Pardo, F. A., Baseggio, A. M., Barbero, G. F., Maróstica Junior, M. R., Rostagno, M. A., & Martínez, J. (2017). Extraction of phenolic compounds and anthocyanins from juçara (Euterpe edulis Mart.) residues using pressurized liquids and supercritical fluids. *The Journal of Supercritical Fluids, 119*, 9–16. http://dx.doi.org/10.1016/j.supflu.2016.08.014.

Kamali, H., Khodaverdi, E., Hadizadeh, F., & Ghaziaskar, S. H. (2016). Optimization of phenolic and flavonoid content and antioxidants capacity of pressurized liquid extraction from Dracocephalum kotschyi via circumscribed central composite. *The Journal of Supercritical Fluids, 107*, 307–314. http://dx.doi.org/10.1016/j.supflu.2015.09.028.

Kim, B. C., Kim, H. G., Lee, S. A., Lim, S., Park, E. H., Kim, S. J., & Lim, C. J. (2005). Genipin-induced apoptosis in hepatoma cells is mediated by reactive oxygen species/c-Jun NH2-terminal kinase-dependent activation of mitochondrial pathway. *Biochemical Pharmacology, 70*(9), 1398–1407. http://dx.doi.org/10.1016/j.bcp.2005.07.025.

Koo, H. J., Lim, K. H., Jung, H. J., & Park, E. H. (2006). Anti-inflammatory evaluation of gardenia extract, geniposide and genipin. *Journal of Ethnopharmacology, 103*(3),

496–500. http://dx.doi.org/10.1016/j.jep.2005.08.011.
Koo, H. J., Song, Y. S., Kim, H.-J., Lee, Y.-H., Hong, S.-M., Kim, S.-J., ... Park, E.-H. (2004). Antiinflammatory effects of genipin, an active principle of gardenia. *European Journal of Pharmacology, 495*(2–3), 201–208. http://dx.doi.org/10.1016/j.ejphar.2004.05.031.
Lee, S. W., Lim, J. M., Bhoo, S. H., Paik, Y.-S., & Hahn, T.-R. (2003). Colorimetric determination of amino acids using genipin from Gardenia jasminoides. *Analytica Chimica Acta, 480*(2), 267–274. http://dx.doi.org/10.1016/S0003-2670(03)00023-0.
Machado, A. P. D. F., Pasquel-Reátegui, J. L., Barbero, G. F., & Martínez, J. (2015). Pressurized liquid extraction of bioactive compounds from blackberry (Rubus fruticosus L.) residues: a comparison with conventional methods. *Food Research International, 77*, 675–683. http://dx.doi.org/10.1016/j.foodres.2014.12.042.
Nithya, P., & Madhavi, C. (2017). Antioxidant activity of 3-arylidene-4-piperidones in the 1,1-diphenyl-2-picrylhydrazyl scavenging assay. *Journal of Taibah University for Science, 11*(1), 40–45. http://dx.doi.org/10.1016/j.jtusci.2014.11.007.
Oliveira, V. B., Yamada, L. T., Fagg, C. W., & Brandão, M. G. L. (2012). Native foods from Brazilian biodiversity as a source of bioactive compounds. *Food Research International, 48*(1), 170–179. http://dx.doi.org/10.1016/j.foodres.2012.03.011.
Omena, C. M. B., Valentim, I. B., Guedes, G.d. S., Rabelo, L. A., Mano, C. M., Bechara, E. J. H., ... Goulart, M. O. F. (2012). Antioxidant, anti-acetylcholinesterase and cytotoxic activities of ethanol extracts of peel, pulp and seeds of exotic Brazilian fruits. *Food Research International, 49*(1), 334–344. http://dx.doi.org/10.1016/j.foodres.2012.07.010.
Osorio-Tobón, F. J., Carvalho, P. I. N., Rostagno, M. A., Petenate, A. J., & Meireles, M. A. A. (2014). Extraction of curcuminoids from deflavored turmeric (Curcuma longa L.) using pressurized liquids: process integration and economic evaluation. *The Journal of Supercritical Fluids, 95*, 167–174. http://dx.doi.org/10.1016/j.supflu.2014.08.012.
Osorio-Tobón, J. F., & Meireles, M. A. A. (2013). Recent applications of pressurized fluid extraction: curcuminoids extraction with pressurized liquids. *Food and Public Health, 3*(6), 289–303. http://dx.doi.org/10.5923/j.fph.20130306.05.
Porto, R. G. C. L., Cardoso, B. V. S., Barros, N. V.d. A., Cunha, E. M. F., Araújo, M. A.d. M., & Moreira-Araújo, R. S.d. R. (2014). Chemical composition and antioxidant activity of *Genipa Americana* L. (Jenipapo) of the Brazilian Cerrado. *Journal of Agriculture and Environmental Sciences, 3*(4), http://dx.doi.org/10.15640/jaes.v3n4a4.
Prosky, L., & Lee, S. (1996). Dietary fiber and its applications: definitions, analytical methods and their applications. *Special Publications of the Royal Society of Chemistry, 181*, 303–310.
Ramos-de-la-Peña, A. M., Montañez, J. C., Reyes-Vega, M.d.l. L., Hendrickx, M. E., & Contreras-Esquivel, J. C. (2015). Recovery of genipin from genipap fruit by high pressure processing. *LWT - Food Science and Technology, 63*(2), 1347–1350. http://dx.doi.org/10.1016/j.lwt.2015.04.038.
Ramos-de-la-Peña, A. M., Renard, C. M. G. C., Montañez, J. C., Reyes-Vega, M.d.l. L., & Contreras-Esquivel, J. C. (2015). Ultrafiltration for genipin recovery technologies after ultrasonic treatment of genipap fruit. *Biocatalysis and Agricultural Biotechnology, 4*(1), 11–16. http://dx.doi.org/10.1016/j.bcab.2014.09.009.
Renhe, I. R. T., Stringheta, P. C., Silva, F. F.e., & Oliveira, T. V.d. (2009). Obtenção de corante natural azul extraído de frutos de jenipapo. *Pesquisa Agropecuária Brasileira, 44*, 649–652.
Rufino, M.d. S. M., Alves, R. E., de Brito, E. S., Pérez-Jiménez, J., Saura-Calixto, F., & Mancini-Filho, J. (2010). Bioactive compounds and antioxidant capacities of 18 non-traditional tropical fruits from Brazil. *Food Chemistry, 121*(4), 996–1002. http://dx.doi.org/10.1016/j.foodchem.2010.01.037.
Ruiz-Aceituno, L., García-Sarrió, M. J., Alonso-Rodriguez, B., Ramos, L., & Sanz, M. L. (2016). Extraction of bioactive carbohydrates from artichoke (Cynara scolymus L.) external bracts using microwave assisted extraction and pressurized liquid extraction. *Food Chemistry, 196*, 1156–1162. http://dx.doi.org/10.1016/j.foodchem.2015.10.046.
Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). *[14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods in enzymology, vol. 299*, Academic Press152–178.
Souza, V. R., Pereira, P. A. P., Queiroz, F., Borges, S. V., & Carneiro, J. (2012). Determination of bioactive compounds, antioxidant activity and chemical composition of Cerrado Brazilian fruits. *Food Chemistry, 134*(1), 381–386. http://dx.doi.org/10.1016/j.foodchem.2012.02.191.
Thomas, P., & Farrugia, K. (2013). An investigation into the enhancement of fingermarks in blood on paper with genipin and lawsone. *Science & Justice, 53*(3), 315–320. http://dx.doi.org/10.1016/j.scijus.2013.04.006.
Vazquez-Roig, P., & Picó, Y. (2015). Pressurized liquid extraction of organic contaminants in environmental and food samples. *TrAC Trends in Analytical Chemistry, 71*, 55–64. http://dx.doi.org/10.1016/j.trac.2015.04.014.
Viganó, J., Aguiar, A. C., Moraes, D. R., Jara, J. L. P., Eberlin, M. N., Cazarin, C. B. B., ... Martínez, J. (2016). Sequential high pressure extractions applied to recover piceatannol and scirpusin B from passion fruit bagasse. *Food Research International, 85*, 51–58. http://dx.doi.org/10.1016/j.foodres.2016.04.015.
Xiao, W., Li, S., Wang, S., & Ho, C.-T. (2017). Chemistry and bioactivity of Gardenia jasminoides. *Journal of Food and Drug Analysis, 25*(1), 43–61. http://dx.doi.org/10.1016/j.jfda.2016.11.005.
Zhang, Y., Yin, F., Liu, J., Liu, Z., Guo, L., Xia, Z., & Zidichouski, J. (2015). Geniposide attenuates insulin-deficiency-induced acceleration of beta-amyloidosis in an APP/PS1 transgenic model of Alzheimer's disease. *Neurochemistry International, 89*, 7–16. http://dx.doi.org/10.1016/j.neuint.2015.04.002.
Zhou, W.-E., Zhang, Y., Li, Y., Ling, Y., Li, H.-N., Li, S.-H., ... Zhang, F. (2016). Determination of gardenia yellow colorants in soft drink, pastry, instant noodles with ultrasound-assisted extraction by high performance liquid chromatography-electrospray ionization tandem mass spectrum. *Journal of Chromatography A, 1446*, 59–69. http://dx.doi.org/10.1016/j.chroma.2016.03.051.

- *CHAPTER 5* -

Obtaining a new natural blue colorant for food industry: Optimization of extraction parameters and economic analysis

Extraction of natural blue colorant from *Genipa americana* L. using green technologies: Techno-economic evaluation

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Article published in the journal Food and Bioproducts Processing, vol. 114., p. 132-143, 2019

ISSN: 0960-3085. DOI: 10.1016/j.fbp.2018.12.004

Received 31 August 2018, Revised 29 November 2018, Accepted 11 December 2018

Food and Bioproducts Processing 114 (2019) 132–143

Contents lists available at ScienceDirect

Food and Bioproducts Processing

journal homepage: www.elsevier.com/locate/fbp

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ARTICLE INFO

Article history:
Received 31 August 2018
Received in revised form 29 November 2018
Accepted 11 December 2018
Available online 16 December 2018

Keywords:
Color additives
Pressurized liquid extraction
Solvent extraction
Pressing extraction
Cost of manufacturing
Genipin

ABSTRACT

The use of green technologies for food production has increased in the last years since they allow obtaining safe products for human consumption. This study reports the optimization of the extraction of the natural blue colorant genipin from genipap fruit using pressurized liquid extraction (PLE), low-pressure extraction (LPSE), and pressing followed by LPSE (Press + LPSE). The effects of the extracting solvent (water and ethanol), temperature (40, 50 and 60 °C) and pressure (0.1, 2, 5 and 8 MPa) on the extraction yield and genipin recovery were investigated. An extensive economic evaluation of the processes was also performed. The results showed that only the extracting solvent influenced extraction yields and genipin recovery. Kinetic curves demonstrated that it was possible to recover 90% of the genipin in a very short time (less than 6 min) by Press + LPSE. Press + LPSE also demonstrated a great economic feasibility with a payback time shorter than 1 year.

1. Introduction

The food industry has been replacing synthetic additives with natural ones due to the modern consumer's demand for healthier products. Among the additives used in the food industry, colorants stand out because color is one of the main attributes evaluated by consumers when deciding to purchase a food product. Currently, synthetic colorants are recognized as carcinogenic and allergenic products and are being widely rejected (Martins et al., 2016).

Brazil has a large diversity of plants that can be used to obtain assorted colors, but there is a scarcity of natural colorants with blue color. In this sense, unripe genipap (*Genipa americana* L.), a native fruit from Brazil rich in genipin appears as a natural source for obtaining the blue color. Genipin is an iridoid that reacts spontaneously with primary amine groups of amino acids, peptides or proteins in the presence of oxygen to form dark-blue pigments (Ramos-de-la-Peña et al., 2014; Náthia-Neves and Meireles, 2018).

For many years, the use of genipin as a colorant was limited to only few Asian countries, such as Japan and Korea, which allow the use of genipin from *Gardenia jasminoides* Ellis fruits as food coloring (Lee et al., 2003). The non-use of this natural colorant in foodstuffs is mainly due to strict safety requirements in Europe and United States. However, this scenario has changed since genipin colorant has been reported as a "fruit juice" color additive in the United States (Title 21 CFR, Code of Federal Regulations, § 73.250) (FDA, 2009) and, more recently, the use of genipin colorant for food was approved in Colombia (Brauch et al., 2016).

Despite of the increased interest for these products, studies investigating the extraction of blue colorant from genipap and its economic viability are still scarce in literature. Nowadays, to be competitive in the current market, processes to obtain pigment-rich products must be not only efficient but also relatively cheap to enable its economic feasibility (Alcázar-Alay et al., 2017). Factors such as performance (obtaining

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https://doi.org/10.1016/j.fbp.2018.12.004
0960-3085/

as much product as possible), productivity (requiring the least amount of processing time) and selectively (obtaining a product rich in the substance of interest) should be considered when determining the economic viability of a process (Prado et al., 2011).

Therefore, the purpose of this work was to obtain a genipin-rich extract with blue color using the peeled genipap fruit as raw material and to optimize the extraction with moderate/low pressures in order to provide a technically and economically feasible process. In this sense, the economic feasibility of the process was determined through the estimation of the cost of manufacturing (COM), revenue, payback time and productivity parameters. To the best of our knowledge, it is the first time that the genipin production is economically evaluated to state its commercial feasibility.

2. Materials and methods

2.1. Raw material preparation

Unripe genipap fruits were acquired from Sítio do Bello (Paraibuna, Brazil) in November 2016. The fruits were separated from peel with a knife and crushed into small particles in a mixer (Philips Walita 400 W, RI1364/07, Varginha, Brazil). After these processes, the raw material was characterized according to moisture (method 920.151 from AOAC, 1997); ash (method 923.03 from AOAC, 1997); protein (method 970.22 from AOAC, 1997); lipids (method from Bligh and Dyer, 1959) and carbohydrates (calculated by difference). The density of the fruit was measured using a glass pycnometer.

2.2. Extraction processes

Fig. 1 shows the paths followed in this study to optimize the genipin extraction from peeled genipap fruit.

2.2.1. Pressurized liquid extraction (PLE)

All extraction assays were performed in a homemade unit described and validated by Johner and Meireles (2016) (Fig. 2a). The parameters evaluated for PLE were the solvent (water and ethanol), temperature (40, 50 and 60 °C) and pressure (0.1, 2, 5 and 8 MPa).

For each assay, 10 g (wet basis, w.b.) of raw material were placed in a 100-mL stainless-steel extraction vessel with a metal filter on the bottom. The void volume was filled with glass beads. The extraction vessel was heated in a heating bath (Thermo Haake, DC30/DL30, Eindhoven, Netherlands). Next, the extraction vessel was filled with a solvent using an HPLC pump (Thermoseparation Products, California, USA) until the desired pressure was reached, and the pressure was maintained for 5 min for the static period. The solvent feed rate was 2 mL/min. After the static time, the blocking valve (Autoclave Engineers, 10 V2071, Pennsylvania, USA) and micrometric valve (Autoclave Engineers, 10 VRM2812, Pennsylvania, USA) were opened and carefully adjusted to maintain the system pressure. The solvent-to-feed mass ratio (S/F) was 5 (w.b.). Global yield (X_0) was calculated as the ratio of the total extract obtained from the extraction and the amount of raw material used on a dry basis.

2.2.2. Extraction kinetics study

2.2.2.1. Low-pressure solvent extraction (LPSE) kinetic. The kinetic experiments used to construct the overall extraction curve (OEC) were performed under the optimal extraction conditions to maximize the extraction yield and genipin recovery. As discussed in the results section, the best results were obtained using water as the solvent at 40 °C and 0.1 MPa, i.e., ambient pressure, and thus, the codes for the process will be substituted using the conventional acronym for low-pressure solvent extraction, LPSE. The solvent feed rate was 8 mL/min, and the mass of the raw material used was 40 g (w.b.). The extraction process was performed as described in Section 2.2.1. An extraction time of 127 min was adopted to ensure that the diffusion-controlled period was reached. The kinetic experiments were replicated 2 times.

2.2.2.2. Press + LPSE (Pressing followed by LPSE) kinetic. At this stage, a mechanical press was used to obtain a concentrated genipin extract without the use of a solvent. The diameter of the press was 19.8 mm. The pressure exerted by the piston on the plant matrix was 67 MPa and was controlled by a torque wrench (SATA, ST96304SC, Sorocaba, Brazil). The press (Fig. 2b) was connected to the system, and the pressing lasted approximately one and a half minutes. After pressing, the extraction was performed as described in Section 2.2.2.1. The kinetic experiments were replicated 2 times.

2.2.2.3. OEC modeling. The experimental data obtained from the OECs were fitted to a three-lines spline model using the PROREG procedure with SAS 9.2® software followed by the NLIN procedure according to Meireles (2008). The fitted lines were attributed to three different steps based on classic descriptions of the periods: the constant extraction rate (CER, Eq. (1)), falling extraction rate (FER, Eq. (2)) and diffusion-controlled (DC, Eq. (3)).

$$\text{for } t \le t_1 : m_{Ext}(t) = b_0 + a_1 t \quad (1)$$

$$t_{CER} \le t \le t_{FER} : m_{Ext}(t) = (b_0 - t_1 a_2) + (a_1 + a_2)\, t \quad (2)$$

$$\text{for } t \ge t_{FER} : m_{Ext}(t) = (b_0 - t_1 a_2 - t_2 a_3) + (a_1 + a_2 + a_3)\, t \quad (3)$$

where m_{Ext} is the extracted mass; t is the extraction time; b_0 is the linear coefficient of the CER line; a_1, a_2 and a_3 are the slopes of the CER, FER and DC lines, respectively; t_{CER} is the CER time span; and t_{FER} is the end of the FER period.

2.3. Extract analyses

2.3.1. Genipin quantification

Genipin content in the extracts was quantified by an HPLC-DAD (Waters, Alliance E2695, Milford, USA) according to Náthia-Neves et al. (2018). The genipin was separated in a fused-core C18 column (Kinetex, 100 × 4.6 mm i.d.; 2.6 μm; Phenomenex, Torrance, USA) using a mobile phase of water (A) and acetonitrile (B) that were both acidified with 0.1% formic acid and the following gradient: 0 min, 99% A; 9 min, 75% A; 10 min, 99% A and 13 min, 99% A. The temperature and flow rate were 35 °C and 1.5 mL/min, respectively. Fig. 3 shows the chromatograms of the standards and the extracts obtained from *G. americana* L.

2.3.2. Color analysis

Color was measured in a Hunterlab colorimeter (Hunter Associates Laboratory, Inc., Reston, Virginia, USA) equipped with a D65 light source with an angle of observation of 2° for all the samples. The extract color was analyzed at room temperature, and the sampling was performed in triplicate.

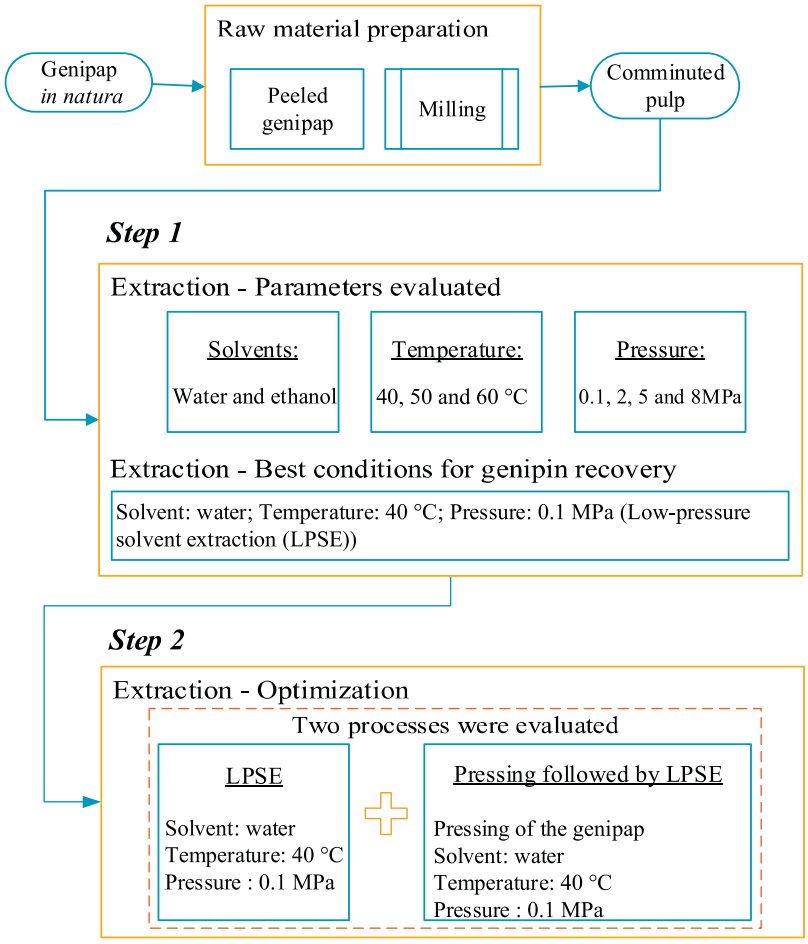

Fig. 1 – Flowchart of the optimization study of the genipin extraction.

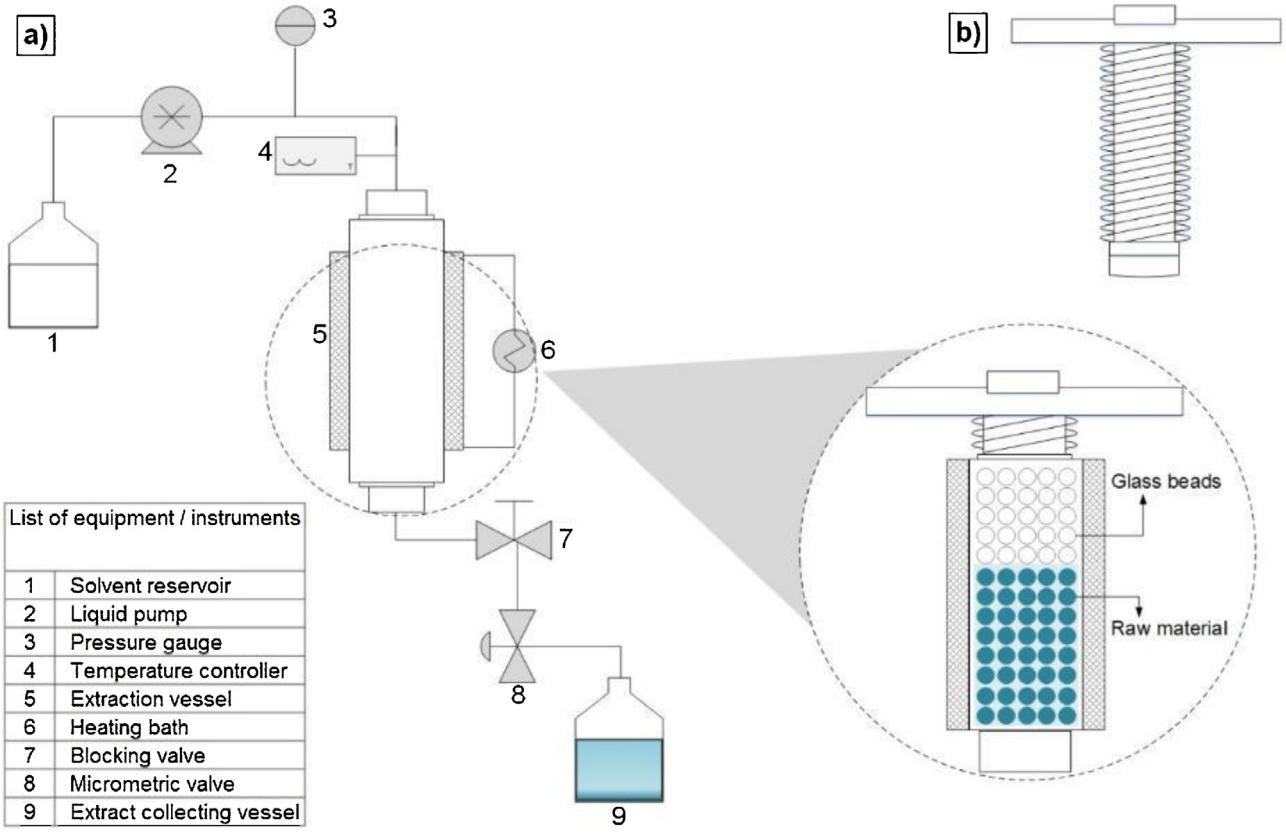

Fig. 2 – (a) Experimental apparatus used for the extraction; (b) the press.

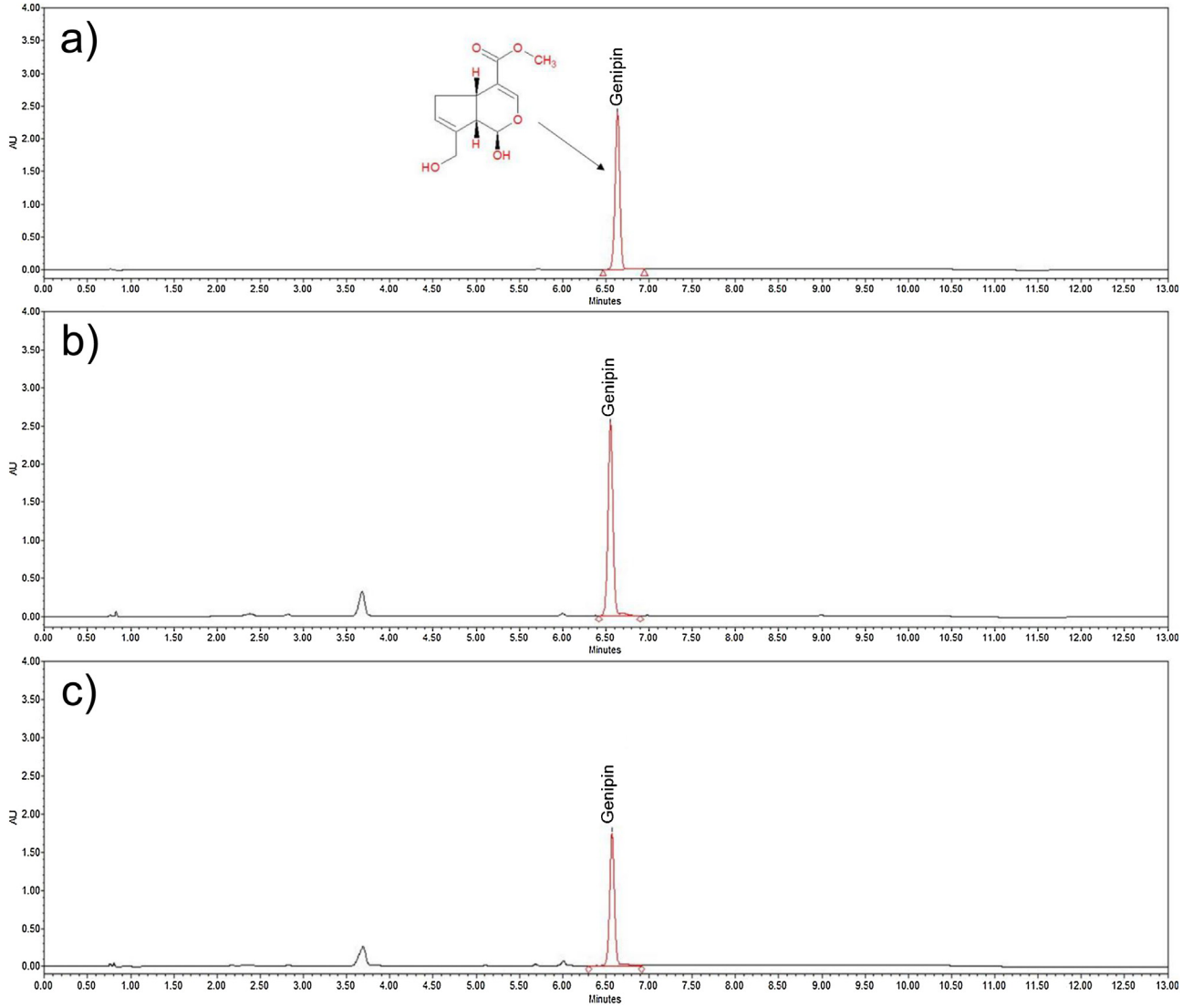

Fig. 3 – Representative HPLC/DAD chromatograms for genipin analysis: (a) standard solution of genipin (625 μg/mL); (b) aqueous extract from *Genipa americana* L. obtained at 40 °C and 0.1 MPa; (c) ethanolic extract from *Genipa americana* L. obtained at 40 °C and 0.1 MPa. Retention time of genipin: 6.6 min.

2.4. *Statistical analysis*

The analysis of the influence of the parameters on the X_0 and genipin recovery of the extracts was performed by analysis of variance (ANOVA) using Minitab 16® software (Minitab Inc., State College, Pennsylvania, USA) with a 95% confidence (p-value ≤0.05).

2.5. *Economic evaluation*

The models of the LPSE and Press + LPSE processes were built using the commercial simulator SuperPro Designer® version 8.5 (Intelligen Inc., Scotch Plains, USA). The input parameters and process conditions were determined according to the kinetic assays. The experimental data used to simulate the LPSE and Press + LPSE are presented in Table 1. The dollar quotation to estimate the costs of the local items was R$ 3.17.

Cost estimation for the equipment on different scales were performed using the power law (Eq. (4)) (Smith, 2005), where: C_1 is the equipment cost with capacity Q_1; C_2 is the known base cost for equipment with capacity Q_2; and n is a constant depending on equipment type (Green and Perry, 2007; Smith, 2005; Turton et al., 2009). The base costs of the equipment used in this work were based on an operating plant with two extractors of 1 L (Table 2).

$$C_1 = C_2\left(\frac{Q_2}{Q_1}\right)^n \quad (4)$$

Table 1 – Experimental data for the process simulations.

Equipment settings	LPSE	Press + LPSE	Unit
Extraction pressure	0.1	0.1	MPa
Extraction temperature	40	40	°C
S/F	4.0	1.5	kg solvent/kg feed (w.b.)
Extraction time	25	7	min
Extraction yield	7.8	6.8	%
Extract genipin content	19.6	21.6	% (w.b.)
Final extract moisture after drying	3.3	1.7	%

Table 2 – Base equipment costs.

Equipment	Unit base cost (US$)[a]	n[b]	LPSE plant		Press + LPSE plant	
			Number of equipment/instruments	Total base cost (US$)[a]	Number of equipment/instruments	Total base cost (US$)[a]
Jacketed extraction vessel[c]	6270.00	0.82	2	12540.00	2	12540.00
Heating bath	2063.09	0.59	1	2063.09	1	2063.09
Electric liquid pump	3920.00	0.55	1	3920.00	1	3920.00
Dryer	8000.00	0.59	1	8000.00	1	8000.00
Press[d]	8479.50	0.59	0	0.00	2	16959.00
Micrometer valve	1090.00	0.6	2	2180.00	2	2180.00
Block valve	220	0.6	4	880.00	4	880.00
Safety valve	310	0.6	2	620.00	2	620.00
Piping, connectors, crossheads, mixers and splitter	3660.00	0.6	1	3660.00	1	3660.00
Structural material for supporting the equipment	4060.00	0.6	1	4060.00	1	4060.00
Manometer	410	–	2	820.00	2	820.00
Temperature controller	310	0.6	2	620.00	2	620.00
Total				39363.09		56322.09

[a] Based on an operating plant with two 1-L extractors from Osorio-Tobón et al. (2016) and Viganó et al. (2017).
[b] *n* constant depending on the equipment type based on Green and Perry (2007), Silla (2003), Smith (2005) and Turton et al. (2009).
[c] Supporting pressures up to 60 MPa.
[d] Direct quotation.

Equipment costs in the scales studied were calculated by Eq. (4) using the base cost data presented in Table 2. Thus, the total costs of the LPSE plants at 10-L, 50-L and 100-L scales were US$ 121401.06, US$ 337767.58 and US$ 529424.31, respectively. The total cost of the LPSE + Press plants at 10-L, 50-L and 100-L scales were US$ 130270.55, US$ 360691.61 and US$ 563930.63, respectively. The annual depreciation rate considered was 10%, and the annual maintenance rate was US$ 6.00/h. The number of workers needed for the 10-L and 50-L plants was two and for the 100-L plant three. The hourly cost of each worker was US$ 13.80 (SuperPro cost database). The cost of utilities was taken from the SuperPro database, and the chilled water cost was US$ 0.40/t, the steam cost was US$ 12.00/t, and the electricity cost was US$ 0.10/kW h. The water cost was US$ 0.05/t.

The cost of manufacturing (COM) was calculated as the ratio between the annual cost of operation and the annual production using the cost tool in the simulator SuperPro Designer® (Intelligen Inc., Scotch Plains, USA). The profitability indices evaluated were return on investment (ROI), payback time, gross margin (GM), net present value (NPV) and internal rate of return (IRR) after taxes, as described by Vardanega et al. (2017a).

2.5.1. *Sensitivity analysis*

The sensitivity analyses were accomplished to explore the uncertainties related to the prices and costs assumed to evaluate the process. Thus, two acquisition costs for genipap were evaluated: US$ 1.42/kg (usual price of genipap in the region where the fruit is largely produced) and US$ 7.89/kg (price of genipap in regions far from the production region). As extracts obtained from genipap are not yet commercialized, it is difficult to establish a selling price for this product. Thus, a sensitivity study was performed with five selling prices: (i) US$ 50.00/kg; (ii) US$ 100.00/kg; (iii) US$ 150.00/kg; (iv) US$ 200.00/kg and (v) US$ 250.00/kg. The range of selling prices evaluated was selected based on extracts obtained from *G. jasminoides* that are commercialized in Asian countries.

Table 3 – Proximate composition (% w/w) of unripe genipap fruit.

Parameter	Results
Moisture	81.02 ± 0.02%
Ash	5.1 ± 0.1%
Lipids	4.0 ± 0.4%
Protein	6.9 ± 0.2%
Carbohydrates	84%
True density	1006.5 ± 0.1 kg/m^3

3. Results and discussion

3.1. *Unripe genipap characterization*

Table 3 presents the proximate composition of unripe genipap fruit. These results are in agreement with that found by Alcázar-Alay et al. (2017), El-Halwagi (2017), Johner and Meireles (2016) and Náthia-Neves et al. (2017). The authors observed that the genipap fruit with peel presented 80% moisture, 5% ash, 7% protein, 3% lipids and 84.7% carbohydrates. Data for the whole fruit without peel were not found in the literature. The mesocarp of the unripe genipap analyzed by Bentes et al. (2015) presented 80.9% moisture, 4.97% ash, 3.24% proteins, 1.52% lipids, 41.19% total fiber and 49% carbohydrates on a dry basis. The same authors found that the endocarp + seeds from unripe genipap fruits presented 68% moisture, 2.75% ash, 9.97% proteins, 1.69% lipids, 46.05% total fiber and 39.54% carbohydrates on a dry basis.

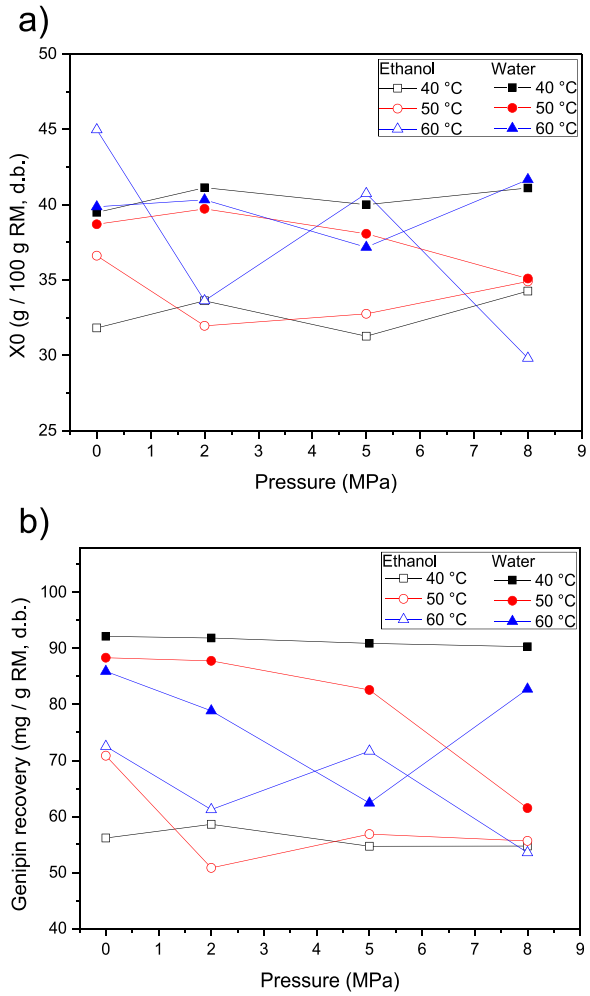

Fig. 4 – Isotherms obtained at different pressures: (a) global yield (X_0); (b) genipin recovery. Standard deviations of the X_0 and genipin recovery from ANOVA ($\alpha = 0.05$) were 4 and 11, respectively.

3.2. *Effect of the process parameters on X_0 and genipin recovery*

The analysis of variance (ANOVA, $\alpha = 0.05$) showed that only the solvent significantly affected the X_0 (p-value = 0.024) and genipin recovery (p-value = 0.001). Fig. 4 shows the isotherms obtained under the different extraction conditions. The X_0 ranged from 30 to 45% (d.b.). A larger X_0 was observed using water as a solvent, which may be related to the high content of carbohydrates (84%), that are easily extracted with water (Vardanega et al., 2017b). In a previous study, it was observed that temperature had a significant effect on X_0 and genipin recovery when 50 and 80 °C were studied; the increase of temperature resulted in a decrease of genipin recovery (Náthia-Neves et al., 2017). However, the results obtained in this study indicated that variations up to 10 °C in the process temperature had no influence on the X_0 and genipin recovery. Therefore, 40 °C was selected since it requires less energy consumption.

The non-influence of the pressure on the extraction using liquid solvents has already been reported by some authors (Vardanega et al., 2017c; Viganó et al., 2016). This occurs because liquids are not compressible fluids. According to Osorio-Tobón and Meireles (2013), even under large pressure changes, the solvation power of the solvent is not significantly affected. However, the use of a higher pressure can favor the extraction of compounds located inside the matrix pores because the pressure forces the solvent to penetrate places that are normally not reached by the solvent at atmospheric pressure (Osorio-Tobón and Meireles 2013). As no significant effect of pressure was observed for the genipin recovery it suggests that genipin is located in cells that are easily accessible by the solvent at lower pressures. From the point of industrial application, processes involving low pressure are very interesting because the equipment are of simple operation, has low cost and the product can be obtained in a food grade (Silva et al., 2017).

The genipin recovery ranged from 51 to 92 mg/g RM (raw material). The highest genipin recovery (92 mg/g RM) was obtained at 40 °C and 0.1 MPa using water as solvent, what is in agreement with the expected, since genipin is an iridoid of polar characteristic (Balamurugan et al., 2014; Wu and Horn, 2015). In the previous study, Náthia-Neves et al. (2017) obtained an extraction yield of 36% (d.b.) and a genipin recovery of 47 mg/g RM at 50 °C and 2 MPa using ethanol as solvent and unripe genipap (whole fruit with peel) as raw material. Although this result was similar to those observed in the present study, the color of the extracts was yellow-like.

Ramos-de-la-Peña et al. (2015) extracted genipin from genipap fruit using the HPP method at 130 MPa with water as solvent and obtained a genipin recovery of 34 ± 2 mg/g RM (w.b.) after 15 min of processing. The recovery reported by these authors was 3 times lower than that obtained in the present study, which can be attributed to the different parts of the genipap used as raw material in each study: these authors used the genipap fruit without seeds while the peeled fruit was used in the present study. Furthermore, the principle of the extraction processes was also different, since the HPP is based on the hydrostatic pressure while the extraction condition that resulted the highest genipin recovery in this study is based on the dynamic contact between the solvent and the raw material.

The highest genipin recovery (196 mg/g RM, d.b) reported in the literature was obtained by Bellé et al. (2018) by an enzyme-assisted extraction in liquid–liquid aqueous system. The best condition was using the Celluclast 10% enzyme at 36 °C and pH of 3.7. Although the genipin recovery was 2.1 times superior than the obtained in this study, the processing time was 4 times longer. These authors also studied the chitosan gels crosslinked with genipin 0.5% and observed better textural and similar rheological properties when compared to the chitosan crosslinked with glutaraldehyde 3%, which makes the genipin an alternative to the use of glutaraldehyde in chitosan crosslinking applications.

3.3. *Color*

Genipin is known to be a natural blue colorant, this colorant is the cross-linked form of genipin (with proteins or amino acids). In this sense the color analyses was performed in order to confirm if the extracts obtained from genipap fruit presented the blue color in all evaluated conditions. The color parameters of the extracts are presented in Table 4. All extracts showed coloration in the blue region (b* negative). The ethanolic extracts showed lighter coloration than the aqueous extracts. The increase in temperature favored a darker

Table 4 – Color parameters for the unripe genipap fruit extracts.

Solvents	T (°C)	P (MPa)	L*	a*	b*	C*	H*
Ethanol	40	0.1	4.3 ± 0.1	−0.33 ± 0.04	−3.2 ± 0.1	3.2 ± 0.1	264.1 ± 0.8
		2	11.7 ± 0.2	−1.4 ± 0.1	−1.7 ± 0.1	2.2 ± 0.1	231 ± 2
		5	11.9 ± 0.6	−2.7 ± 0.4	−2.8 ± 0.2	3.9 ± 0.3	226 ± 5
		8	27.39 ± 0.01	−0.6 ± 0.1	−2.23 ± 0.02	2.3 ± 0.02	256 ± 1
	50	0.1	5.6 ± 0.1	−0.65 ± 0.01	−3.9 ± 0.1	4 ± 0.1	260.7 ± 0.3
		2	10.8 ± 0.1	−1.2 ± 0.1	−1.3 ± 0.1	1.83 ± 0.06	227 ± 4
		5	10.7 ± 0.2	−1.7 ± 0.1	−1.3 ± 0.2	2.1 ± 0.1	217 ± 5
		8	9.6 ± 0.1	−1.7 ± 0.1	−2 ± 0.2	2.7 ± 0.1	230 ± 4
	60	0.1	4.1 ± 0.2	−1.12 ± 0.01	−3.9 ± 0.1	4.0 ± 0.1	255 ± 3
		2	8.9 ± 0.8	−2.1 ± 0.2	−3.2 ± 0.2	3.8 ± 0.1	237 ± 4
		5	7.8 ± 0.9	−1.3 ± 0.1	−3.9 ± 0.3	4.2 ± 0.3	252 ± 2
		8	10 ± 0.3	−2 ± 0.1	−3.6 ± 0.2	4.2 ± 0.2	241 ± 2
Water	40	0.1	24.84 ± 0.01	−0.38 ± 0.02	−1.47 ± 0.02	1.51 ± 0.02	255.6 ± 0.8
		2	7.36 ± 0.04	−1 ± 0.1	−2.4 ± 0.1	2.6 ± 0.1	247 ± 2
		5	8.11 ± 0.03	−1 ± 0.1	−2.1 ± 0.2	2.3 ± 0.1	244 ± 4
		8	7.59 ± 0.03	−1.7 ± 0.1	−3 ± 0.1	3.4 ± 0.1	241 ± 2
	50	0.1	3.48 ± 0.04	−1.1 ± 0.1	−2.74 ± 0.05	2.9 ± 0.05	248.2 ± 0.9
		2	5.6 ± 0.02	−1.24 ± 0.06	−2.9 ± 0.1	3.194 ± 0.06	247 ± 1
		5	5.71 ± 0.02	−1.4 ± 0.1	−1.5 ± 0.1	2.04 ± 0.05	227 ± 3
		8	5.3 ± 0.03	−1.8 ± 0.1	−1.9 ± 0.1	2.71 ± 0.08	227 ± 2
	60	0.1	3.72 ± 0.04	−1.15 ± 0.05	−2.88 ± 0.05	3.10 ± 0.05	248 ± 1
		2	4.61 ± 0.03	−1.1 ± 0.1	−3.8 ± 0.1	4.01 ± 0.05	254 ± 1
		5	3.55 ± 0.04	−1.14 ± 0.05	−1.88 ± 0.05	2.35 ± 0.03	233 ± 2
		8	3.91 ± 0.02	−1.1 ± 0.1	−3.97 ± 0.04	4.12 ± 0.04	255 ± 1

L*: luminosity: black (L* = 0) and white (L* = 100); a*: green color (−) and red color (+); b*: blue color (−) and yellow color (+); C*: chroma; H*: hue angle.

coloration in both solvents, and the aqueous extract at 60 °C presented a color close to black (low L*). These results confirmed that the extracts obtained from unripe genipap fruits can be used as a colorant by the food industry and supply a natural blue color currently lacking in the food industry.

At this stage of the study, it was possible to select the extraction condition that resulted the extract with the highest genipin recovery and blue color to proceed the kinetic study in order to evaluate the kinetic behavior of the extraction. The extraction parameters selected were: 40 °C and 0.1 MPa, i.e., ambient pressure, using water as solvent.

3.4. *Extraction kinetics*

Overall extraction curves (OECs) based on the extraction yield and genipin recovery are presented in Fig. 5. As an alternative for increasing the genipin recovery with the minimal consumption of solvent, a mechanical pressing step was added before the solvent extraction to obtain a concentrated aqueous extract fraction. After pressing, the solvent extraction was carried out to recover the remain genipin in the raw material. Thus, next it will be presented the comparison of the OECs obtained without and with the pressing step, named LPSE and Press + LPSE, respectively. As discussed previously, the extraction performed with LPSE (40 °C and 0.1 MPa) using an S/F of 5 and an extraction time of approximately 25 min allowed an extract yield of 40 ± 2% and a genipin recovery of 92.2 ± 0.4 mg/g RM. The extraction using only pressing (first point of Fig. 5a and b) allowed an extract yield of 26 ± 2% and a genipin recovery of 52 ± 10 mg/g RM in a time of 1.19 min.

The higher extraction yield obtained in the LPSE process (Fig. 5a) can be explained by the fact that other compounds have been extracted from the vegetable matrix (probably carbohydrates) over time, while the pressing compaction caused in the Press + LPSE may have hampered the extraction of other compounds from the plant matrix. Regarding the genipin recovery, at the end of the OECs the genipin amount was the same for both processes (LPSE and Press + LPSE), which shows that both processes were able to deplete all genipin present in the raw material (Fig. 5b).

The OECs obtained for the LPSE and Press + LPSE processes presented similar behaviors with three characteristic stages: CER, FER and DC periods. Higher amounts of extract and genipin were obtained in the CER and FER periods. However, it is possible to observe in Fig. 5a that the Press + LPSE reached the CER period faster than LPSE because pressing may favored the mass transfer rate in this period. For the recovery of genipin, although the CER and FER times of LPSE and Press + LPSE processes were close, the genipin recovery in the CER period of the Press + LPSE was higher than that of the LPSE (Fig. 5b). In addition, the curves obtained by the Press + LPSE presented a DC (diffusion controlled) period more pronounced than the curves obtained from the LPSE. This indicates that the use of the press greatly contributed to the removal of compounds from the vegetable matrix.

From the fitted data, it was possible to estimate the parameters of the LPSE and Press + LPSE processes, as shown in Table 5. The t_{CER} for LPSE (8.1 ± 0.9 min) was higher than the t_{CER} for Press + LPSE (5.89 ± 0.03 min). The extraction yields obtained at these times for these processes were 30 ± 3% and 36 ± 3%, respectively. These yields correspond to 64% of the total yield of the LPSE (after 127 min) and 91% of the total yield of the Press + LPSE (after 128 min). These results are consistent with those reported in the literature, where approximately 50–90% of the total extract amount was obtained in the CER period (Pereira and Meireles, 2009). This occurs because in the CER period the solute is easily accessible and solubilized in the solvent (Soares et al., 2016). Although the t_{FER} values for the LPSE and Press + LPSE processes were similar, the yield obtained at this time in the LPSE (43 ± 4%) was higher than the yield at that time in the Press + LPSE (38 ± 3%). These yields

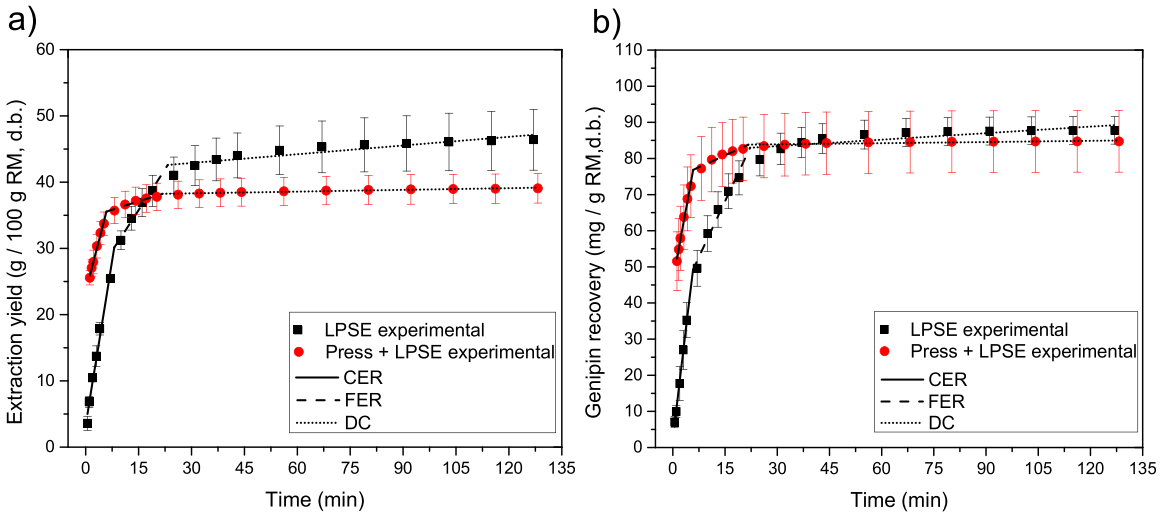

Fig. 5 – Overall extraction curves obtained at 40 °C and 0.1 MPa using water as the solvent: (a) extraction yield; (b) genipin recovery. The error bars represent the amplitude which is the difference between the lowest and highest value divided by two.

Table 5 – Kinetic parameters estimated by the spline model for the extraction yield and genipin recovery.

Extraction yield (d.b.)		
Kinetic parameters	LPSE	Press + LPSE
t_{CER} (min)	8.1 ± 0.9	5.89 ± 0.03
R_{CER} (%)	30 ± 3	36 ± 3
M_{CER} x 10^6 (kg/s)	4.23 ± 0.03	2.7 ± 0.2
Y_{CER} x 10^2 (kg extract/kg water)	3.2 ± 0.3	2.0 ± 0.2
R^2	1	1
t_{FER} (min)	23.3 ± 0.2	21.8 ± 0.3
R_{FER} (%)	43 ± 4	38 ± 3
M_{FER} x 10^6 (kg/s)	1.0 ± 0.1	0.3 ± 0.2
Y_{FER} x 10^3 (kg extract/kg water)	7.5 ± 0.1	2.5 ± 0.2
R^2	0.978	1
Genipin recovery (d.b.)		
Kinetic parameters	LPSE	Press + LPSE
t_{CER} (min)	5.8 ± 0.6	5.84 ± 0.02
R_{CER} (%)	4.9 ± 0.6	8 ± 1
M_{CER} x 10^6 (kg/s)	1.0 ± 0.1	0.7 ± 0.1
Y_{CER} x 10^3 (kg genipin/kg water)	7.5 ± 0.1	5.0 ± 0.1
R^2	0.999	1
t_{FER} (min)	22.2 ± 0.2	21.7 ± 0.4
R_{FER} (%)	8.3 ± 0.6	8 ± 1
M_{FER} x 10^7 (kg/s)	1.7 ± 0.1	0.5 ± 0.1
Y_{FER} x 10^3 (kg genipin/kg water)	1.3 ± 0. 1	0.4 ± 0.1
R^2	1	0.999

d.b. = dry basis; t_{CER} = duration of constant extraction rate period; R_{CER} = yield for CER period; M_{CER} = mass transfer rate for CER period; Y_{CER} = mass ratio of solute in fluid phase at extractor outlet for CER period; t_{FER} = duration of falling extraction rate period; R_{FER} = yield for FER period; M_{FER} = mass transfer rate for FER period; Y_{FER} = mass ratio of solute in fluid phase at extractor outlet for FER period.

correspond to 90% of the total yield of LPSE (after 127 min) and 98% of the total yield of the Press + LPSE (after 128 min).

For genipin recovery using LPSE, t_{CER} resulted in a recovery of only 55% of the total genipin and t_{FER} resulted in 93% of the total genipin, while for the Press + LPSE, 90% of the total genipin was recovered in the t_{CER}. Therefore, the LPSE process should be performed for 22.2 min (t_{FER}), while the Press + LPSE process should be performed for 5.84 min (t_{CER}).

3.5. *Economic evaluation*

Although the Press + LPSE demonstrated that the processing time could be reduced from 22.2 min to approximately 5.84 min, it required the addition of an additional unit operation to the process, which in turn represent an additional cost to the equipment acquisition. To evaluate these impact on the economic feasibility of the genipin production, a detailed economic evaluation was performed.

3.5.1. *Influence of scale-up on the COM and productivity*

The LPSE and Press + LPSE processes were simulated to determine the COM of the extract from unripe genipap fruit and the productivity and total capital investment for different extraction vessel volumes (2 × 10 L, 2 × 50 L and 2 × 100 L) and raw material costs (US$ 1.42/kg and US$ 7.89/kg), and the results are shown in Fig. 6. Two raw materials costs were considered for this simulation to evaluate the impact of this variable on economic viability of the processes because the acquisition cost vary drastically depending on the genipap production region in Brazil.

Considering the raw material cost of US$ 1.42/kg (Fig. 6a), the COM ranged from US$ 49.36/kg to US$ 95.03/kg in the LPSE process and from US$ 46.28/kg to US$ 80.34/kg in the Press + LPSE process. When the raw material cost of US$ 7.89/kg (Fig. 6b) was considered, the cost inherent to raw material acquisition exerted a strong influence on the manufacturing costs. The estimated COM ranged from US$ 129.63/kg to US$ 175.30/kg for the LPSE and US$ 140.08/kg to US$ 174.15/kg for the Press + LPSE.

As observed in Fig. 6, the COM decreases with the increase in the production scale. The same behavior was reported for the extraction of carotenoids from pressed palm fibers using LPSE (Cardenas-Toro et al., 2015), for the extraction of curcuminoids from deflavored turmeric using PLE (Osorio-Tobón et al., 2014) and for the extraction of phenolic compounds from jabuticaba skins using PLE (Santos et al., 2012). The extract productivity obtained in the Press + LPSE process was 1.3 times higher than that in the LPSE process. The higher productivity of the Press + LPSE process is related to its shorter process time, which allows more batches per year than the PLE process. The

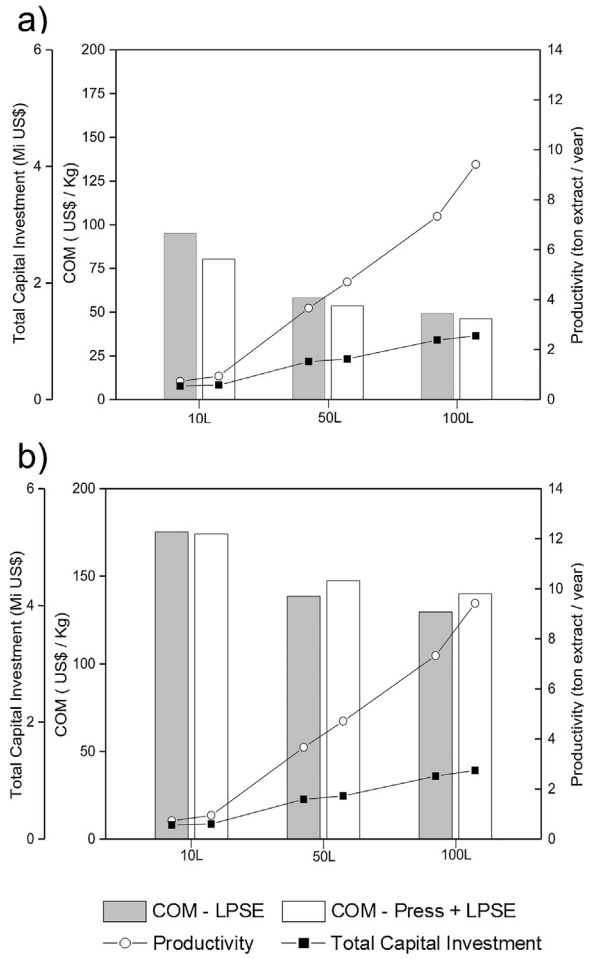

Fig. 6 – Influence of the system capacity on the COM, productivity and total capital investment of the LPSE and Press + LPSE processes: (a) based on the cost of raw material of US$ 1.42/kg; (b) based on the cost of raw material of US$ 7.89/kg.

total cost of investment showed little variation between the two processes.

The COM was calculated considering the costs of the raw material, facilities, labor and utilities (Carvalho et al., 2015). The percent contribution of the economic parameters to the COM for the two raw material costs in the five plant capacities studied are presented in Fig. 7. For the cost of raw material of US$ 1.42/kg, the facility components represent the largest contribution to the COM in both LPSE and Press + LPSE processes. As the scale increased, the contribution of labor and facility components to the COM was reduced, which indicated the feasibility of the processes on larger scales. In contrast, the contribution of raw materials increased as the scale increased.

Raw materials are generally the components with the highest contribution to the COM (Osorio-Tobón et al., 2016). This can be easily observed by increasing the cost of raw material to US$ 7.89/kg (Fig. 7). In this situation, the raw material becomes the component with the greatest contribution to the COM for both LPSE and Press + LPSE processes. The influence of the cost of raw material in the Press + LPSE is greater than that in the LPSE because this process occurs more rapidly, and therefore, it requires a greater amount of raw material to be processed. The change in the raw material cost from US$ 1.42/kg to US$ 7.89/kg represents increases of 46% (10 L), 58% (50 L) and 62% (100 L) for the total COM for the LPSE process and increases of 54% (10 L), 64% (50 L) and 67% (100 L) for the total COM for the Press + LPSE process.

3.5.2. *Sensitivity analysis*

As genipin is not yet commercialized as a colorant worldwide, it is difficult to predict its selling price. Thus, to evaluate the influence of the extract selling price on the feasibility of the process, a sensitivity analysis was performed using selling prices from US$ 50.00 to 250.00/kg for the extract. Tables 6 and 7 present an executive summary of the project indices, which were calculated for the LPSE and Press + LPSE processes at the 100-L scale, respectively. The project indices for the 10 L and 50 L scales are presented in the Supplementary material.

The gross margin (GM) is an economic indicator used to estimate the short-term benefits of a specific activity. This indicator is calculated as the ratio between the annual profits and the annual revenues and is expressed as a percentage (Vlysidis et al., 2011). A higher GM indicates a more attractive project. In Tables 6 and 7, with a raw material cost of US$

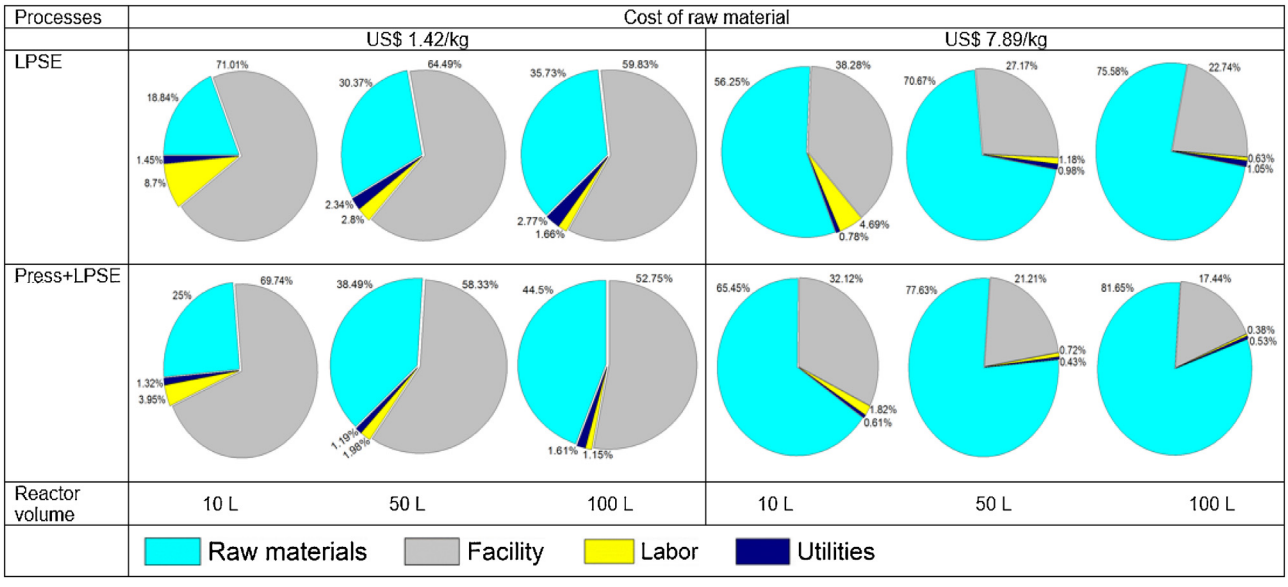

Fig. 7 – Composition of the COM for the LPSE and Press + LPSE processes with different raw material costs.

Table 6 – Project indices of the LPSE process at a 100-L scale.

Selling price (US$/kg)	GM (%)	ROI (%)	Payback time (year)	IRR (%)	NPV (US$) (at 7% interest)
Raw material cost = 1.42 US$/kg					
50.00	−2.13	7.79	12.84	N/A	−42200.00
100.00	48.94	28.83	3.47	22.42	1121000.00
150.00	65.96	49.57	2.02	38.05	2647000.00
200.00	74.47	70.32	1.42	51.17	4173000.00
250.00	79.57	91.07	1.10	62.27	5682000.00
Raw material cost = 7.89 US$/kg					
50.00	−168.24	−47.18	N/A	N/A	−4576000.00
100.00	−34.12	−14.32	N/A	N/A	−2067000.00
150.00	10.59	14.36	6.96	8.36	87000.00
200.00	32.94	34.08	2.93	27.11	1613000.00
250.00	46.35	53.79	1.86	41.48	3139000.00

NA: not applicable; ROI: return on investment; IRR: internal rate of return after taxes; NPV: net present value.

Table 7 – Project indices of the Press + LPSE process at a 100-L scale.

Selling price (US$/kg)	GM (%)	ROI (%)	Payback time (year)	IRR (%)	NPV (US$) (at 7% interest)
Raw material cost = 1.42 US$/kg					
50.00	5.85	9.97	10.03	2.11	−272000.00
100.00	52.92	35.34	2.83	27.89	1713000.00
150.00	68.62	60.72	1.65	45.39	3706000.00
200.00	76.46	86.09	1.16	59.77	5685000.00
250.00	81.17	111.46	0.90	72.11	7651000.00
Raw material cost = 7.89 US$/kg					
50.00	−185.01	−64.97	N/A	N/A	−6453000.00
100.00	−42.50	−25.58	N/A	N/A	−3176000.00
150.00	5.00	11.45	8.73	5.23	−115000.00
200.00	28.75	35.09	2.85	28.20	1856000.00
250.00	43.00	58.72	1.70	45.08	3849000.00

NA: not applicable; ROI: return on investment; IRR: internal rate of return after taxes; NPV: net present value.

1.42/kg, the GM demonstrates positive values for the selling price of extracts higher than US$ 100.00 for the LPSE process and for all selling prices for the Press + LPSE process. Using a raw material cost of US$ 7.89/kg, the GM presents positive values only for selling prices above US$ 150.00 for both the LPSE and Press + LPSE processes.

The return on investment (ROI) is the percentage of money recovered annually from the plant's profit, and thus, a higher ROI indicates a more desirable product (Vlysidis et al., 2011). In general, a minimum ROI of 5–10% is necessary to accept a project (El-Halwagi, 2017; Fernández-Ronco et al., 2013). For a raw material cost of US$ 1.42/kg, all evaluated scenarios presented acceptable values of ROI, except for a selling price of US$ 50.00/kg for the extract using LPSE. However, for a raw material cost of US$ 7.89/kg, selling prices above US$ 150.00/kg for the extract were necessary to make the processes feasible.

Payback time represents the time required to recover the cost of investment. Clearly, shorter payback times are more attractive; payback times between 2 and 5 years are considered feasible. As expected, the best payback occurs with higher selling prices and lower raw material costs for both processes. The Press + LPSE process showed slightly lower payback times than the LPSE process. However, for both processes, using a raw material cost of US$ 1.42/kg, selling prices above US$ 100.00/kg for the extract are required to make the processes feasible. However, for a raw material cost of US$ 7.89/kg, only selling prices above US$ 150.00/kg for the extract are feasible to reach acceptable payback times.

The net present value (NPV) represents the difference between the present value of cash inflows and the present value of cash outflow. If the NPV of a project is positive after assuming an interest rate of 7% (Osorio-Tobón et al., 2016), it can be considered feasible. Furthermore, to undertake a project, the internal rate of return after taxes (IRR) should be as high as possible because it represents the rate of return at which the project's NPV is zero (Vucurovic et al., 2012). Therefore, both processes became feasible at selling prices higher than US$ 100.00/kg for the extract with a raw material cost of US$ 1.42/kg; for the cost of raw material of US$ 7.89/kg, selling prices above US$ 150.00/kg for the extract are needed.

It is worth to mention that the processes developed in this study result products safe for food application and can be recognized as green processes, since they use GRAS (Generally Recognized as Safe) solvents, requires a low energy consumption and the biomass generated at the end of the processes can be reused for the production of biofilms, animal feed or even to produce bioenergy (Chemat et al., 2017). Furthermore, the processes are economically viable because they enable obtaining a high yield of genipin that is considered a compound of high added value for industry.

4. Conclusion

The results found in this work demonstrated that the peeled unripe genipap is a suitable source of a blue color extract and that it was possible to reduce the processing time

from 22.2 min to 5.84 min by adding a pressing step to the low-pressure solvent extraction method. From an economic standpoint, both processes are applicable to industrial scales. The raw material cost and selling price of the extract were demonstrated to greatly affect the feasibility of the process. Thus, the present work showed the commercial potential of genipap fruit and provide future perspectives for the food industry since a natural blue colorant was obtained in a short time.

Author contributions

Grazielle Náthia-Neves: Collected the experimental data and drafted the manuscript.

Renata Vardanega: Performed the economic analysis.

Maria Angela de Almeida Meireles: Designed the study and helped with the results interpretation.

Acknowledgments

G. Náthia-Neves thanks CAPES (Finance Code 001) for a Ph.D. assistantship. R. Vardanega thanks CAPES (Finance Code 001) for the postdoctoral assistantship, and M.A.A. Meireles thanks CNPq for the productivity grant (302423/2015-0).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fbp.2018.12.004.

References

Alcázar-Alay, S.C., Osorio-Tobón, J.F., Forster-Carneiro, T., Meireles, M.A.A., 2017. Obtaining bixin from semi-defatted annatto seeds by a mechanical method and solvent extraction: process integration and economic evaluation. Food Res. Int. 99, 393–402.

AOAC, 1997. Official methods of analysis of the Association of Official Analytical Chemistry. AOAC International, Gaithersburg, USA.

Balamurugan, M., Rajesh, S., Manogaran, E., 2014. 'Genipin' – the natural water soluble cross-linking agent and its importance in the modified drug delivery systems: an overview. Curr. Drug Deliv. 11, 139–145.

Bellé, A.S., Hackenhaar, C.R., Spolidoro, L.S., Rodrigues, E., Klein, M.P., Hertz, P.F., 2018. Efficient enzyme-assisted extraction of genipin from genipap (*Genipa americana* L.) and its application as a crosslinker for chitosan gels. Food Chem. 246, 266–274.

Bentes, Adria de S., de Souza, Hugo A.L., Amaya-Farfan, Jaime, Lopes, Alessandra S., de Faria, Lênio J.G., 2015. Influence of the composition of unripe genipap (*Genipa americana* L.) fruit on the formation of blue pigment. J. Food Sci. Technol. 52 (6), 3919–3924.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Brauch, J.E., Zapata-Porras, S.P., Buchweitz, M., Aschoff, J.K., Carle, R., 2016. Jagua blue derived from *Genipa americana* L. fruit: a natural alternative to commonly used blue food colorants? Food Res. Int. 89, 391–398.

Cardenas-Toro, F.P., Alcázar-Alay, S.C., Coutinho, J.P., Godoy, H.T., Forster-Carneiro, T., Meireles, M.A.A., 2015. Pressurized liquid extraction and low-pressure solvent extraction of carotenoids from pressed palm fiber: experimental and economical evaluation. Food Bioprod. Process. 94, 90–100.

Carvalho, P.I.N., Osorio-Tobón, J.F., Rostagno, M.A., Petenate, A.J., Meireles, M.A.A., 2015. Techno-economic evaluation of the extraction of turmeric (*Curcuma longa* L.) oil and ar-turmerone using supercritical carbon dioxide. J. Supercrit. Fluids 105, 44–54.

Chemat, F., Rombaut, N., Meullemiestre, A., Turk, M., Perino, S., Fabiano-Tixier, A.-S., Abert-Vian, M., 2017. Review of green food processing techniques. Preservation transformation, and extraction. Innovative Food Sci. Emerg. Technol. 41, 357–377.

El-Halwagi, M.M., 2017. Sustainable design through process integration: fundamentals and applications to industrial pollution prevention, resource conservation, and profitability enhancement. Butterworth-Heinemann.

FDA, 2009. Food and Drugs Administration – listing of color additives exempt from certification; food, drug and cosmetic labeling: cochineal extract and carmine declaration (21 CFR parts 73 and 101). Federal register 74 (2), 207–217.

Fernández-Ronco, M.P., de Lucas, A., Rodríguez, J.F., García, M.T., Gracia, I., 2013. New considerations in the economic evaluation of supercritical processes: separation of bioactive compounds from multicomponent mixtures. J. Supercrit. Fluids 79, 345–355.

Green, D., Perry, R., 2007. Perry's Chemical Engineers' Handbook, eighth ed. McGraw-Hill Education.

Johner, J.C.F., Meireles, M.A.A., 2016. Construction of a supercritical fluid extraction (SFE) equipment: validation using annatto and fennel and extract analysis by thin layer chromatography coupled to image. Food Sci. Technol. (Campinas) 36, 210–247.

Lee, S.-W., Lim, J.-M., Bhoo, S.-H., Paik, Y.-S., Hahn, T.-R., 2003. Colorimetric determination of amino acids using genipin from *Gardenia jasminoides*. Anal. Chim. Acta 480, 267–274.

Martins, N., Roriz, C.L., Morales, P., Barros, L., Ferreira, I.C.F.R., 2016. Food colorants: challenges, opportunities and current desires of agro-industries to ensure consumer expectations and regulatory practices. Trends Food Sci. Technol. 52, 1–15.

Meireles, M.A.A., 2008. Extraction of bioactive compounds from Latin American plants. Supercritical fluid extraction of nutraceuticals and bioactive compounds, 243–274.

Náthia-Neves, G., Meireles, M.A.A., 2018. Genipap: a new perspective on natural colorants for the food industry. Food Public Health 8, 21–33.

Náthia-Neves, G., Tarone, A.G., Tosi, M.M., Maróstica Júnior, M.R., Meireles, M.A.A., 2017. Extraction of bioactive compounds from genipap (*Genipa americana* L.) by pressurized ethanol: iridoids, phenolic content and antioxidant activity. Food Res. Int. 102, 595–604.

Náthia-Neves, G., Nogueira, G.C., Vardanega, R., Meireles, M.A.A., 2018. Identification and quantification of genipin and geniposide from *Genipa americana* L. by HPLC-DAD using a fused-core column. Food Sci. Technol. (Campinas), in press.

Osorio-Tobón, F.J., Carvalho, P.I.N., Rostagno, M.A., Petenate, A.J., Meireles, M.A.A., 2014. Extraction of curcuminoids from deflavored turmeric (*Curcuma longa* L.) using pressurized liquids: process integration and economic evaluation. J. Supercrit. Fluids 95, 167–174.

Osorio-Tobón, J.F., Carvalho, P.I.N., Rostagno, M.A., Meireles, M.A.A., 2016. Process integration for turmeric products extraction using supercritical fluids and pressurized liquids: economic evaluation. Food Bioprod. Process. 98, 227–235.

Osorio-Tobón, J.F., Meireles, M.A.A., 2013. Recent applications of pressurized fluid extraction: curcuminoids extraction with pressurized liquids. Food Public Health 3, 289–303.

Pereira, C.G., Meireles, M.A.A., 2009. Supercritical fluid extraction of bioactive compounds: fundamentals, applications and economic perspectives. Food Bioprocess Technol. 3, 340–372.

Prado, J.M., Prado, G.H.C., Meireles, M.A.A., 2011. Scale-up study of supercritical fluid extraction process for clove and sugarcane residue. J. Supercrit. Fluids 56 (3), 231–237.

Ramos-de-la-Peña, A.M., Montañez, J.C., de la LuzReyes-Vega, María., Hendrickx, M.E., Contreras-Esquivel, J.C., 2015. Recovery of genipin from genipap fruit by high pressure processing. LWT – Food Sci. Technol. 63, 1347–1350.

Ramos-de-la-Peña, A.M., Renard, C.M., Wicker, L., Montanez, J.C., Garcia-Cerda, L.A., Contreras-Esquivel, J.C., 2014.

Environmental friendly cold-mechanical/sonic enzymatic assisted extraction of genipin from genipap (*Genipa americana*). Ultrason. Sonochem. 21, 43–49.
Santos, D.T., Veggi, P.C., Meireles, M.A.A., 2012. Optimization and economic evaluation of pressurized liquid extraction of phenolic compounds from jabuticaba skins. J. Food Eng. 108, 444–452.
Silla, H., 2003. Chemical Process Engineering: Design and Economics. Taylor & Francis.
Silva, S., Costa, E.M., Calhau, C., Morais, R.M., Pintado, M.E., 2017. Anthocyanin extraction from plant tissues: a review. Crit. Rev. Food Sci. Nutr. 57, 3072–3083.
Smith, R., 2005. Chemical Process Design and Integration. Wiley, Chichester.
Soares, J.F., Zabot, G.L., Tres, M.V., Lunelli, F.C., Rodrigues, V.M., Friedrich, M.T., Pazinatto, C.A., Bilibio, D., Mazutti, M.A., Carniel, N., Priamo, W.L., 2016. Supercritical CO_2 extraction of black poplar (*Populus nigra* L.) extract: experimental data and fitting of kinetic parameters. J.Supercrit. Fluids 117, 270–278.
Turton, R., Bailie, R.C., Whiting, W.B., 2009. Analysis, synthesis, and design of chemical processes. Prentice Hall.
Vardanega, R., Carvalho, P.I.N., Albarelli, J.Q., Santos, D.T., Meireles, M.A.A., 2017a. Techno-economic evaluation of obtaining Brazilian ginseng extracts in potential production scenarios. Food Bioprod. Process. 101, 45–55.
Vardanega, R., Carvalho, P.I.N., Santos, D.T., Meireles, M.A.A., 2017b. Obtaining prebiotic carbohydrates and beta-ecdysone from Brazilian ginseng by subcritical water extraction. Innovative Food Sci. Emerg. Technol. 42, 73–82.
Vardanega, R., Santos, D.T., Meireles, M.A.A., 2017c. Proposal for fractionating Brazilian ginseng extracts: process intensification approach. J. Food Eng. 196, 73–80.
Viganó, J., Brumer, I.Z., Braga, P.A.d.C., da Silva, J.K., Maróstica Júnior, M.R., Reyes Reyes, F.G., Martínez, J., 2016. Pressurized liquids extraction as an alternative process to readily obtain bioactive compounds from passion fruit rinds. Food Bioprod. Process. 100, 382–390.
Viganó, J., Zabot, G.L., Martínez, J., 2017. Supercritical fluid and pressurized liquid extractions of phytonutrients from passion fruit by-products: economic evaluation of sequential multi-stage and single-stage processes. J. Supercrit. Fluids 122, 88–98.
Vlysidis, A., Binns, M., Webb, C., Theodoropoulos, C., 2011. A techno-economic analysis of biodiesel biorefineries: assessment of integrated designs for the co-production of fuels and chemicals. Energy 36, 4671–4683.
Vucurovic, D.G., Dodic, S.N., Popov, S.D., Dodic, J.M., Grahovac, J.A., 2012. Process model and economic analysis of ethanol production from sugar beet raw juice as part of the cleaner production concept. Bioresour. Technol. 104, 367–372.
Wu, S., Horn, G., 2015. Genipin-rich material and its use. Google Patents.

- *CHAPTER 6* -

Biorefinery of genipap: obtaining an extract rich in fatty acids by SFE and a genipin-rich extract by LPSE

Obtaining fatty acids and genipin from *Genipa americana* L. in a biorefinery concept: SFE process integrated with low-pressure solvent extraction

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Manuscript to be submitted for publication in The Journal of Supercritical Fluids

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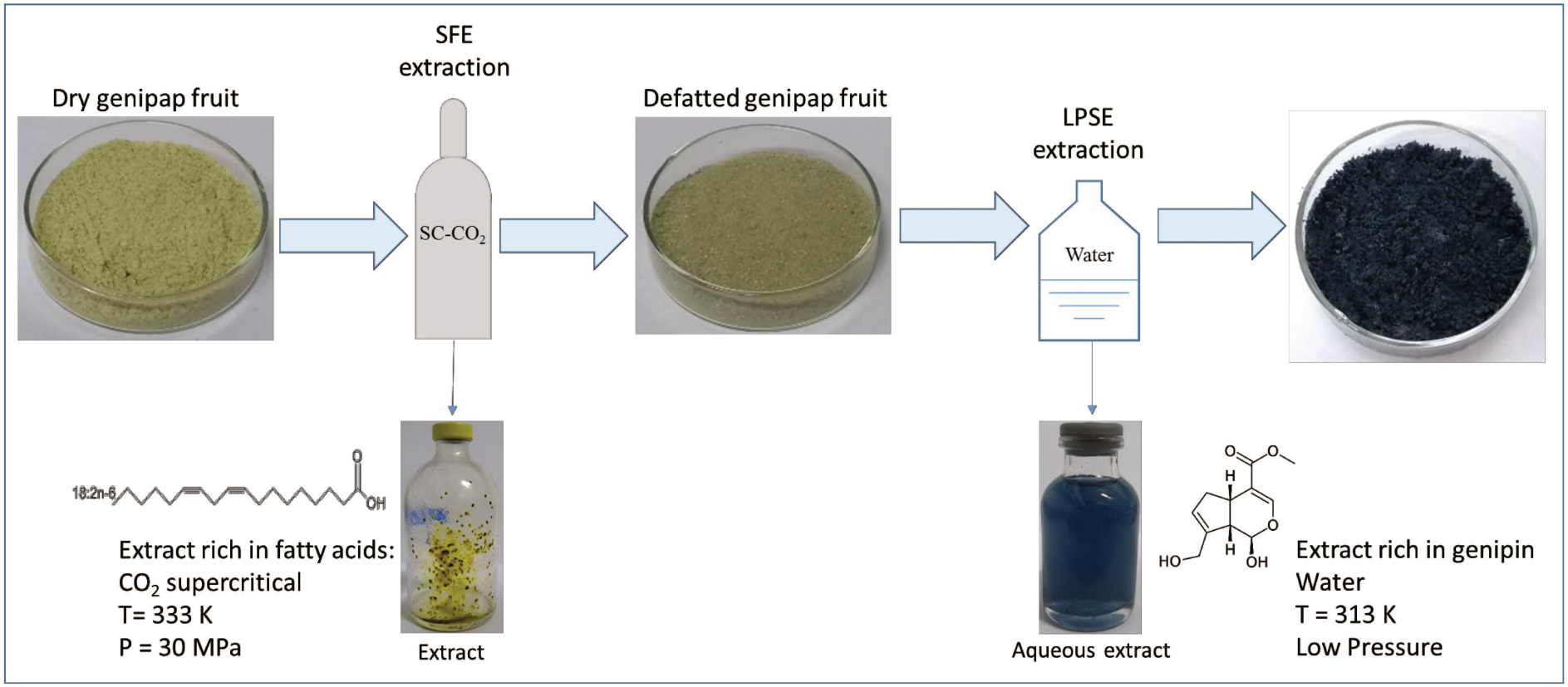
Graphical abstract



Highlights

- The effects of SFE parameters on fatty acid recovery from genipap fruit were evaluated.
- The best operating conditions of SFE in terms of global yield were 30 MPa and 333 K.
- Low-pressure extraction of genipin from defatted unripe genipap fruit was conducted.
- Process integration was useful in obtaining fatty acids and genipin-rich extract.
- The data were accurately modeled based on mass conservation.

Abstract

Supercritical fluid extraction (SFE) using carbon dioxide plays an important role in modern biorefineries since it is a technology that uses sustainable principles to obtain products with high added value. This study was organized in three steps. In the first step, SFE was used for the extraction of the fatty acid-rich extract from unripe genipap fruit. The extract with the highest yield (4.6 ± 0.1%) and total fatty acid content (16.6 mg fatty acids / g of genipap) was obtained at 30 MPa and 333 K. In the second step, the kinetic behavior of the overall extraction curves was studied, and the data were accurately modeled using the potential of the hot sphere diffusion model. Finally, SFE was integrated to low-pressure solvent extraction to obtain a genipin-rich extract (71 mg /g of genipap) from the defatted solid or SFE coproduct.

Keywords: Supercritical fluid extraction, Process integration, Fatty acids, Natural blue colorant, Hot sphere diffusion model.

1. Introduction

Currently, industries in various market segments have increasingly sought to use sustainable processes to obtain their products. Sustainable processes are defined as those that aim to maximize production while minimizing environmental impact by maintaining a productive harmony between humans and nature, thus ensuring the well-being of present and future generations [1]. In this sense, the concept of a biorefinery represents a major advance for industry and the environment. The main goal of this concept is zero residue generation, and in view of this, biorefineries integrate different processes and equipment to obtain products with high added value (chemicals, biofuels, food and feed ingredients, biomaterials, fibers, heat, and power) using several biomasses [2, 3]. Among the existing technologies, those that use green concepts to obtain products sustainably have become increasingly attractive in recent years. Supercritical fluid extraction (SFE) is a promising green technique for obtaining nonpolar compounds, such as fatty acids, essential oils, volatile compounds, and carotenoids, from several vegetable matrices [4]. Carbon dioxide (CO_2) is the main solvent used as a supercritical fluid because it is a safe solvent for food applications and has a low cost and high availability [5]. Upon reaching a supercritical state (304.25 K and 7.39 MPa), CO_2 acquires properties such as gas-like viscosity and diffusivity and liquid-like density that increase the solvation power of the solute, providing a solvent-free product [5, 6].

On the other hand, if the objective is to obtain compounds with polar characteristics, the use of liquid solvents such as water, which is an inexpensive, environmentally friendly, and safe solvent for food application [7], is recommended. Extraction with liquid solvents can occur using pressurized liquid extraction (PLE) or low-pressure solvent extraction (LPSE), according to the characteristics of the raw material and the compound to be extracted. In a previous study, Náthia-Neves, Vardanega

and Meireles [8] observed that the genipin colorant can be efficiently and economically extracted at 313 K and ambient pressure (0.1 MPa) using water as solvent.

Previous studies carried out with several vegetable matrices in different countries show the efficiency of the integration of the productive chain in terms of obtaining high-quality and economically viable products [2, 3, 9-12]. The integration of SFE with other techniques (PLE, LPSE, ultrasound, and supercritical antisolvent (SAS)) has already been studied to maximize the recovery of different compounds from the same raw material [13-17].

Unripe genipap (*Genipa americana* L.) is a little-known fruit that can potentially be explored in an integrated production chain. The main bioactive compound of this fruit is genipin, a powerful natural blue colorant with polar characteristics that, besides coloring, has antioxidant, anticancer and neuroprotective activity and acts against liver diseases [18]. However, this fruit is also a source of nonpolar compounds, such as fatty acids, whose extraction has not yet been explored. The discovery of new sources of these compounds, especially unsaturated fatty acids, is the objective of many researchers because these compounds, also known as essential fatty acids, are essential in the human diet; i.e., they cannot be synthesized by animals, including humans [19, 20]. These acids, in addition to being a reserve source in most organisms, play a variety of cellular functions, such as lowering cholesterol, enhancing brain health, lowering the risk of coronary and fatal heart disease, reducing inflammation and normalizing heart rate variability [21, 22].

Thus, the main goal of this study was to apply the biorefinery concept to the intere use of unripe genipap fruit. For this purpose, the extraction of fatty acids by SFE was experimentally optimized, and a mathematical model was adjusted. Finally, integration

of the SFE-LPSE processes was performed to obtain the fatty acids by SFE in the first step and a genipin-rich extract by LPSE in the second step.

2. Material and Methods

2.1 Sample preparation

Unripe genipap fruits were obtained from the company Sítio do Belo (Paraíbuna, São Paulo, Brazil) in November 2016. The fruits with peel were dried in a freeze-dryer system (Liobras, model L 101, Sao Carlos, Brazil). The dried fruits with peel were ground in a knife mill (Marconi, model MA-340, Piracicaba, Brazil), and the particle size distribution was determined in a vibratory system (Bertel, model 1868, Caieiras, Brazil) using sieves from 16-80 mesh (Tyler series, Wheeling, USA). The mean particle diameter (dp) was determined according to the ASAE method [23]. The ground samples were packed in impermeable plastic bags and stored at 255 K until the extraction assays. The true density of the particles (ρr) was measured by picnometry with helium gas at the Analytical Center of the Institute of Chemistry/UNICAMP (Campinas, Brazil). The apparent density of the bed (ρa) was calculated as the ratio of feed mass to the volume occupied by the sample in the extraction vessel. The total porosity of the bed (ε) was calculated as $\varepsilon = 1 - (\rho a/\rho r)$. The raw material was also characterized using AOAC methods to determine the moisture content (method 920.151 [24]), ash content (method 923.03 [24]), protein content (method 970.22 [24]), and lipid content (method 963.15 [24]); the carbohydrate content was calculated by difference. The assays were performed in triplicate, and the results are expressed as the means ± standard deviations.

2.2 Chemicals

Carbon dioxide (purity > 99.8%), which was used as a solvent for SFE, was supplied by Gama Gases (São Paulo, Brazil). The n-hexane used for the Soxhlet

extraction was obtained from Dinâmica (São Paulo, Brazil) and was of analytical grade. The reagents and solvents used for the conversion of fatty acids to fatty acid methyl esters were sodium hydroxide, supplied by Synth (São Paulo, Brazil); sodium chloride, supplied by Ecibra (São Paulo, Brazil); and boron trifluoride-methanol solution, supplied by Sigma-Aldrich (São Paulo, Brazil), all of analytical grade. The Supelco® FAME (fatty acid methyl ester) Mix C4-C24 used as reference standard and methyl nonadecanoate (purity > 98.0%) used as internal standard were supplied by Sigma-Aldrich (Darmstadt, Germany).

2.3 Oil extraction

2.3.1 Soxhlet extraction

The Soxhlet method was selected as the conventional extraction technique for comparison purposes. Soxhlet extraction was performed according to the protocol of the Association of Official Analytical Chemists (AOAC, 1997). Five grams of sample was wrapped in filter paper and inserted into the Soxhlet apparatus connected to a solvent flask containing 300 mL of hexane. After that, the system was heated to boiling. Reflux was continued for 6 h, and then the solvent was evaporated under vacuum (at 308 K) (Marconi, model MA120, Piracicaba, Brazil). The mass of extract was measured on an analytical balance (Bel, M214Ai, São Paulo, Brazil). The assays were performed in triplicate, and the results of oil yield were expressed as the mean ± standard deviation.

2.3.2 SFE

SFE experimental runs were carried out in a commercial SFE unit (Spe-ed 7071, Applied Separations, Allentown, USA) equipped with a cooling bath (Marconi, model MA184, São Paulo, Brazil), a pneumatic pump, an electric oven, an extraction vessel of 5 mL (Thar Designs, Pittsburgh, PA), a compressor (Shulz S/A, model MS 3, Santa

Catarina, Brazil), and a flow totalizer (LAO G0, São Paulo, Brazil), as shown in Figure 1.

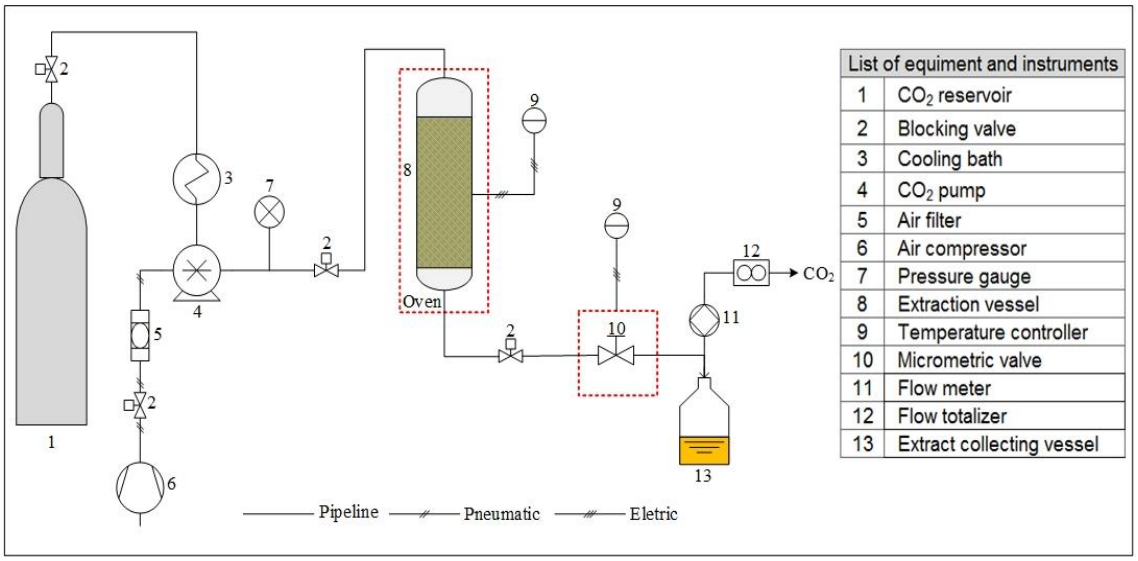


Figure 1. Diagram of the Spe-ed SFE unit.

For the experiments, CO_2 was cooled to 268 K in a thermostatic bath (Marconi, model MA-184, Piracicaba, Brazil) before reaching the pump. The vessel was assembled into the oven, which was maintained at the preselected temperature. The temperature was measured by a thermocouple introduced into the outer wall of the extractor vessel (Pyrotec, TP100, Campinas, Brazil). CO_2 was pumped into the system until reaching the experimental pressure, which was maintained for 5 minutes as the static period. After that, the blocking valve (Autoclave Engineers, model 10 V2071, PA, USA) and micrometer valve (Autoclave Engineers, model 10 VRM2812, PA, USA) were opened and carefully adjusted to maintain the system pressure. The extraction was performed until an S/F ratio of 20 g CO_2/g genipap was reached. For each assay, 3.5 g (dry basis, d.b.) of raw material was placed in the 5-mL stainless-steel extraction vessel, and the CO_2 flow rate was kept constant at 2.5 g/min. The total CO_2 mass was measured by means of the flow totalizer and vented to the ambient. The extracts were collected in glass flasks

submerged in an ice bath, weighed in an analytical balance (Bel, model M214Ai, São Paulo, Brazil), and stored at 255 K for further analyses.

2.4 Overall extraction curves

The kinetics experiments were performed under the optimal conditions with respect to the highest global yield (temperature = 333 K and pressure = 30 MPa). For each kinetic assay, 50 g (d.b.) of raw material was placed in a 300-mL stainless-steel extraction vessel, and the CO_2 flow rate was held constant at 5.6 g/min. The extraction process was performed in the same commercial SFE instrument described in Section 2.3.2. A total extraction time of 400 minutes was adopted to ensure that the diffusion-controlled period would be reached. These experiments were performed in duplicate.

2.5 HSD model

The HSD (hot sphere diffusion) model is based on several simplifying assumptions, namely, spherical single-size particles; no gradient of velocity, pressure, temperature, or solute concentration within the fluid; no sample shrinkage; and a constant diffusion coefficient of extract through particles. Using these hypotheses, the material balance across a particle in the extractor, based on Fick's first law, can be written as follows [25]:

$$\frac{\partial C}{\partial t} = \frac{D_e}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial C}{\partial r}\right) \quad (1)$$

where *C* is the component concentration in the particle, r is the distance from the particle center, and *De* is the effective diffusion coefficient. As this partial differential equation (PDE) is second order with respect to *r*, boundary conditions at two points of the *r* axis are required:

$$at\ r = 0\ \Longrightarrow \frac{\partial C}{\partial r} = 0 \tag{2}$$

$$at\ r = R\ \Longrightarrow C = C^{*} \tag{3}$$

where C^* is the equilibrium concentration of a component in the particle and R is the radius of the particle. The initial condition of the PDE is as follows:

$$at\ t = 0 \Longrightarrow C = C_0 \tag{4}$$

where C_0 is the initial component concentration in the particle. An analytical solution of equation (1) results in [25]:

$$Yield = Yield_{max}\left(1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}exp\left(-\left(\frac{n\pi}{R}\right)^2 D_e t\right)\right) \tag{5}$$

where $Yield$ is the extraction yield in terms of the mass of one specific component per mass of raw material (mg/g), $Yield_{max}$ is the highest extraction yield (mg/g), and n is the counter index of the summation. $Yield_{max}$ and *De* are the two adjustable parameters of this model that should be determined so that the differences between the model and experimental data, in terms of absolute error, can be minimized. In this study, a genetic algorithm [26], which is an efficient optimization technique, was employed for this purpose.

2.6 Integrated SFE and LPSE process

The SFE and LPSE integration was performed to obtain two products, an extract rich in fatty acids and an extract rich in genipin. The extract rich in fatty acids was obtained by SFE according to the parameters optimized in Section 2.4, using the following process conditions: temperature of 333 K, pressure of 30 MPa, and S/F of 16 g of CO_2/g genipap. After SFE, the defatted raw material was transferred to another instrument, where it was submitted to LPSE to obtain a genipin-rich extract. LPSE was performed according to the parameters optimized by Náthia-Neves, Vardanega and Meireles [8]

(temperature = 313 K, flow rate = 2 g / min, and S/F = 20 g of water/g genipap (d.b)). For global yield determination in the LPSE process, a 5 mL aliquot of aqueous extract was evaporated in a vacuum oven (Tecnal, model TE-3951, Piracicaba, Brazil) at 373 K. Then, the global yield was calculated as the ratio of the total extract obtained from the extraction and the amount of raw material used on a dry basis. The assays were performed in duplicate.

2.7 Analytical methods

2.7.1 Fatty acid composition by gas chromatography (GC)

FAMEs were analyzed by gas chromatography (GC-FID) with flame ionization (Shimadzu, model CG17A, Kyoto, Japan) equipped with a fused-silica capillary column ZB-5 (Phenomenex Zebron ZB-5, 30 m × 0.25 mm i.d. × 0.25 μm, USA). The samples were converted to methyl esters by esterification, as described by Joseph and Ackman [27]. Chromatographic separation was carried out according to Pollierer, Dyckmans, Scheu, Haubert and Treseder [28] with some modification. The temperature program started at 343 K (1 min) and increased by 279 K per minute to 583 K (15 min). The split ratio was equal to 1:30. The injection temperature was 523 K, and helium (White Martins, 99.99%) was the carrier gas with a flow rate of 2.2 mL/min. The fatty acid methyl esters were identified by comparison with standards. Quantification was performed by internal normalization using methyl nonadecanoate (1 mg/mL).

2.7.2 Genipin quantification by HPLC analysis

The extracts were analyzed by an HPLC-DAD (Waters, Alliance model E2695, Milford, USA) system. Genipin was separated according to the method described by Náthia-Neves, Nogueira, Vardanega and Meireles [29] using water (A) and acetonitrile (B), both acidified with 0.1% formic acid, as the mobile phase, with the following

gradient: 0 min, 99% A; 9 min, 75% A; 10 min, 99% A and 13 min, 99% A. The temperature and solvent flow rate used were 308 K and 1.5 mL/min, respectively.

2.8 Statistical analyses

The parameters were evaluated with a randomized full factorial design (2 × 5) with temperature (313 and 333 K) and pressure (15, 20, 25, 30 and 35 MPa) in duplicate, resulting in 20 experimental runs (Table 1). The influence of the parameters on global yield and palmitic, stearic, linoleic and linolenic acid contents were evaluated by analysis of variance (ANOVA) using Minitab 16® software (Minitab Inc., State College, PA, USA) with a 95% confidence level (p-value ≤ 0.05).

Table 1. Experimental conditions studied in the SFE of genipap fruit and global yield (X_0) results.

Temperature (K)	Pressure (MPa)	CO_2 density (kg/m^3)	X_0 (%)
313	15	781.32	3.48 ± 0.04
	20	840.61	3.7 ± 0.1
	25	880.15	4 ± 0.1
	30	910.47	4.2 ± 0.2
	35	935.34	4.3 ± 0.2
333	15	605.60	2.5 ± 0.1
	20	724.63	3.4 ± 0.2
	25	787.28	4.1 ± 0.2
	30	830.33	4.6 ± 0.1
	35	863.49	4.6 ± 0.2

X_0 (%) = gram extract / 100 grams of genipap fruit in dry base.
CO_2 density data extracted from Nist (https://webbook.nist.gov/chemistry/fluid/).

3. Results and discussion

3.1 Raw material characterization

The proximate composition of unripe genipap fruit used in the SFE process is shown in Table 2. The high moisture content of the sample acts as a barrier to the diffusion of supercritical carbon dioxide (SC-CO_2) into the matrix as well as the diffusion of oil out

of the matrix, which consequently reduces the SC-CO_2 sample contact [30]. The moisture content for efficient extraction may range from 3 to 24% depending on the raw material used [30]. Therefore, the water content of the unripe genipap fruit after freeze-drying was adequate for the SFE process. The ash, protein, lipid and carbohydrate contents observed in this study are consistent with those found in the literature, ranging from 2.8% to 10.1% for ash, from 1.5% to 10.0% for proteins, from 1.7% to 11.4% for lipids, and from 74% to 91% for carbohydrates [31-34].

Table 2. Proximate composition (% w/w) of unripe genipap fruit in dry basis.

Parameter	Results	Units
Mean particle diameter	0.23 ± 0.03	mm
Real density	1.41 ± 0.01	g/cm^3
Moisture	5.1 ± 0.2	%
Ash	4.16 ± 0.04	%
Protein	7.1 ± 0.5	%
Lipids	8.0 ± 0.6	%
Carbohydrates	80.7	%

The results are the mean ± standard deviation of experiments performed in triplicate.

3.2 Global yield (X_0) and fatty acid composition

The SFE yields from unripe genipap fruit for two isotherms (313 and 333 K) at five pressures (15, 20, 25, 30 and 35 MPa) are shown in Figure 2. Comparing these results with those obtained by Soxhlet extraction revealed that Soxhlet extraction had a higher X_0 (8.0 ± 0.6%). This could be explained by the interaction between solvent recycling and the solvent/solute ratio used in the Soxhlet extraction in addition to the solvent boiling temperature employed in the Soxhlet method, which enhances the solubility of most extractable compounds and provides a higher surface tension and viscosity than SC-CO_2 [35].

The analysis of variance (ANOVA, $\alpha = 0.05$) showed that the pressure (p-value < 0.001) and the interaction between pressure and temperature (p-value < 0.001) significantly influenced X_0 in the studied range. The increase in the operational pressure at a constant temperature resulted in enhancement of X_0, which is mainly related to the increase in CO_2 density, ranging from 781.3 to 935.3 kg/m^3 for 313 K and from 605.6 to 863.5 kg/m^3 for 333 K (Table 1). The three highest yields of 4.3 ± 0.2, 4.6 ± 0.2 and 4.6 ± 0.1% were achieved at densities of 935.3, 863.5 and 830.3 kg / m^3, respectively. The two lowest yields of 2.5 ± 0.1% and 3.48 ± 0.04% were achieved at densities of 605.6 and 781.3 kg / m^3, respectively.

These results are in agreement with a previous publication showing that the solubility of the fatty acids in SC-CO_2 increases with pressure due to enhancement of the solvent solvation power and provides a higher and better permeability of the solvent into the solid matrix [36, 37]. The effect of temperature on X_0 is more complex. At a constant temperature of 313 K, an increase in yield is observed for pressures ranging from 15 to 20 MPa, where the CO_2 density ranged from 781.3 to 840.6 kg/m^3. However, at pressures higher than 25 MPa, an increase in the extraction temperature to 333 K promoted an increase in the X_0 even though there was a reduction in the CO_2 density (787.3 kg/m^3). This behavior is called a crossover pressure and can be defined as the point at which the slope of the solubility versus temperature curve changes sign and the opposite effects of solute vapor pressure and solvent density on solubility compensate for each other [38]. That is, at pressures lower than the crossover pressure, the effect of the density of the CO_2 was more pronounced in the X_0, while at pressures above the crossover pressure, the effect of the vapor pressure with the temperature had a more significant effect than the reduction of the CO_2 density, consequently increasing the X_0. The crossover pressure observed in this study, at which the isotherms of different temperatures (313 and 333 K)

intersect each other, was around of 23.5 MPa. A similar behavior was also reported in previous works for different solid matrices [39-41].

Table 3 shows the fatty acids identified in the extracts obtained by SFE under different conditions of pressure and temperature. The results were expressed in two different ways: as mg of fatty acid per gram of extract, which represents the concentration of fatty acids in the extract, and as mg of fatty acid per gram of raw material, which represents the amount of fatty acid extracted from the unripe genipap fruit. The fatty acids present in all samples were palmitic acid (ranging from 20 to 28 mg / g extract), stearic acid (ranging from 14 to 16 mg / g extract), linoleic acid (ranging from 145 to 310 mg / g extract), and linolenic acid (ranging from 34 to 52 mg / g extract). No significant differences in the profiles were observed; that is, in all the extracts, the same fatty acids were found. Similar observations by Benito-Román, Rodríguez-Perrino, Sanz, Melgosa and Beltrán [42] and dos Santos, de Aguiar, Viganó, Boeing, Visentainer and Martínez [43] indicate that the process parameters of temperature and pressure do not affect the profile of the fatty acids.

The analysis of variance (ANOVA, $\alpha = 0.05$) showed that the pressure and temperature significantly influenced only the linoleic and linolenic acid contents in the studied range. The recovery of linoleic acid was significantly influenced by pressure (p-value < 0.001), temperature (p-value = 0.043) and the interaction between pressure and temperature (p-value = 0.005). The recovery of linolenic acid was significantly influenced by pressure (p-value = 0.006) and temperature (p-value = 0.079).

The highest amount of total fatty acids was observed when the extraction was performed at 333 K and 25 MPa and 30 MPa and resulted in 16.6 and 16.9 mg/g of genipap fruit, respectively. Linoleic acid was the major fatty acid found in the extract from unripe genipap fruit (approximately $76 \pm 1\%$), followed by linolenic acid ($13.3 \pm 0.2\%$), palmitic acid ($7.2 \pm 0.2\%$) and stearic acid ($6.6 \pm 0.6\%$). These results

show that unripe genipap fruit can be used as a good source for obtaining a rich extract of unsaturated fatty acids, such as linoleic and linolenic acids, which are essential to maintaining the integrity of cell membranes, brain function, transmission of nerve impulses, hemoglobin synthesis and cell division [43-46]. As there was no difference in the X_0 or the composition of fatty acids extracted at 30 and 35 MPa at 333 K, it is suggested that extraction from unripe genipap can be carried out at a pressure of 30 MPa and a temperature of 333 K. Although extraction by the Soxhlet method provided a higher extract yield, the amount of fatty acids extracted was lower, which indicates the higher selectivity of SFE compared to Soxhlet.

To the best of our knowledge, no study on the extraction of unripe genipap fruit using SC-CO_2 has yet been reported in the scientific literature. Therefore, the findings of this article were exclusively compared with conventional extraction of ripeness genipap. According to Costa, Ballus, Teixeira Filho and Godoy [47], the genipap pulp extract obtained by shaking presents the following fatty acid profile: palmitic (2.73 ± 0.01%), margaric (20 ± 3%), stearic (0.66 ± 0.01%), oleic (3.7 ± 0.1%), linoleic (35 ± 19%), linolenic (26 ± 16%), behenic (2.27 ± 0%), and lignoceric (1.3 ± 0.2%). Figueiredo, Maia, Holanda and Monteiro [33] studied the oil obtained from the seeds and the pulp of the ripe genipap fruit. The oil obtained from the seeds presented the following fatty acid profile: palmitic (10.29%), stearic (9.74%), oleic (19.48%), and linoleic (60.49%). The oil obtained from pulp presented the following fatty acid profile: capric (2.25%), lauric (2.25%), myristic (5.26%), palmitic (37.2%), stearic (5.36%), and oleic (25.65%). These differences among the fatty acid profiles found in this study and the profiles reported in the literature may be due to variations in the raw materials used for the extraction, which were the pulp and the seed in the studies mentioned above and the whole fruit with the

peel in the present study. Furthermore, the ripeness stage of the fruit may play an important role in the fatty acid profile.

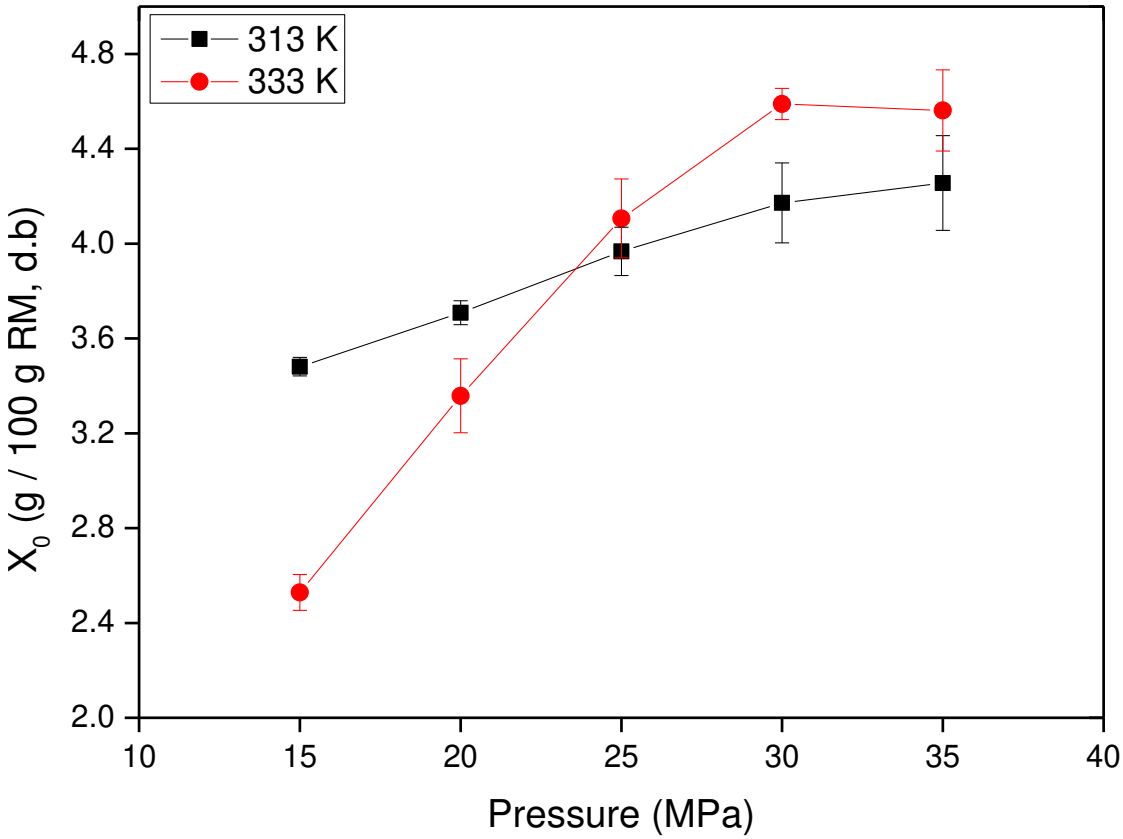


Figure 2. Global yield isotherms from unripe genipap extraction in supercritical carbon dioxide. RM; Raw material; d.b: dry basis.

Table 3. Fatty acid composition of unripe genipap fruit extracts obtained by SFE and Soxhlet.

Fatty acids (mg /g extract)	Soxhlet	Pressure and temperature									
		15 MPa		20 MPa		25 MPa		30 MPa		35 MPa	
		313 K	333 K	313 K	333 K	313 K	333 K	313 K	333 K	313 K	333 K
Palmitic	8.4 ± 0.4	26 ± 1	20 ± 2	26 ± 2	23.4 ± 0.4	26 ± 2	26 ± 14	20 ± 2	24 ± 2	28 ± 2	26 ± 2
Stearic	4.5 ± 0.4	14.8 ± 0.8	14 ± 2	16 ± 2	13.6 ± 0.6	14 ± 2	14 ± 4	16 ± 2	14 ± 2	16 ± 2	16 ± 2
Linoleic	66 ± 3	145 ± 16	196 ± 20	292 ± 16	252 ± 2	192 ± 28	272 ± 74	304 ± 12	276 ± 20	310 ± 26	284 ± 6
Linolenic	10.5 ± 0.3	50 ± 2	34 ± 4	50 ± 2	43.8 ± 0.6	50 ± 4	46 ± 12	52 ± 2	48 ± 4	52 ± 4	50 ± 2
Total	88.9	237.5	264	384	332.8	282	358	392	362	406	366
Fatty acids (mg /g RM)	Soxhlet	Pressure and temperature									
		15 MPa		20 MPa		25 MPa		30 MPa		35 MPa	
		313 K	333 K	313 K	333 K	313 K	333 K	313 K	333 K	313 K	333 K
Palmitic	0.67 ± 0.03	0.88 ± 0.06	0.50 ± 0.02	0.98 ± 0.08	0.8 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	1.04 ± 0.1	1.2 ± 0.1
Stearic	0.36 ± 0.03	0.50 ± 0.04	0.34 ± 0.04	0.6 ± 0.1	0.5 ± 0.1	0.54 ± 0.06	0.6 ± 0.2	0.4 ± 0.2	0.6 ± 0.1	0.60 ± 0.04	0.7 ± 0.1
Linoleic	5.3 ± 0.2	9.8 ± 0.8	4.8 ± 0.8	10.6 ± 0.8	9 ± 2	11 ± 1	13 ± 3	9.4 ± 0.8	13 ± 1	12 ± 2	12.8 ± 0.4
Linolenic	0.84 ± 0.03	1.7 ± 0.2	0.8 ± 0.1	1.8 ± 0.1	1.6 ± 0.2	1.8 ± 0.2	2.2 ± 0.6	1.6 ± 0.2	2.2 ± 0.2	2.0 ± 0.2	2.2 ± 0.1
Total	7.17	12.88	6.44	13.98	12.1	14.44	16.6	12.3	16.6	15.64	16.9

RM: raw material.

3.3 Kinetic extraction curves and modeling

The extraction of a solute from a solid raw material involves three different periods: constant extraction rate (CER), falling extraction rate (FER) and diffusion-controlled (DC) [48]. In this section, the extraction kinetics were analyzed at 333 K and 30 MPa to verify the behavior of the extractions of oil from unripe genipap as a function of extraction time and S/F (Figure 3).

Figure 3a shows classical kinetic curves for obtaining fatty acids using SC-CO_2 [35, 49]. The extraction yield increases rapidly up to the end of the CER period (34 minutes; 3% yield), and the mechanism of mass transfer was mainly controlled by convection in the fluid film around the milled particles. Afterwards, there was a reduction in the extraction rate until the end of the FER period (112 minutes and a yield of almost 5%), which corresponded to the transition period between convection and diffusional extraction. After this time, the extract yield was reduced to 6% after 400 minutes of extraction, which indicates a long diffusional period. The curves did not present the classical plateau during SC-CO_2 extraction, which suggests that either the whole extract content was not extracted from the raw material in 400 minutes or other compounds were extracted due to the long period of contact of the raw material with the solvent inside the bed. However, to reduce the operational time and costs, it is suggested that the extraction of the unripe genipap oil be interrupted at the 112 minutes (S/F = 16), since it was observed that the yield increase is slow along the processing time, and therefore, it would be more advantageous to start a new batch than to continue with the same extraction. This is in agreement with some studies that report that it is preferable to work on the CER period, sometimes extending it to the FER period [49].

The different fatty acids identified showed similar behavior throughout the extraction time. The predominance of linoleic and linolenic acids for the assays studied herein was observed, where the maximum yields of these acids after 400 minutes of extraction were 25 (Figure 3d) and 4.5 mg / g RM (Figure 3e), respectively. The contents

of stearic and palmitic acids were 1.3 (Figure 3c) and 2.5 mg / g RM (Figure 3b), respectively. According to Figure 3f, genipin was also extracted during SFE, although it has low affinity for CO_2. This can be explained by the prolonged effect of the high pressure and the extraction temperature, which may have promoted cell disruption and facilitated mass transfer of the other solutes to the solvent.

The adjustable parameters for the HSD model are presented in Table 4. It was found that using GA with a population size equal to 200 and a generation size equal to 400 can guarantee obtaining reliable $Yield_{max}$ and D_e. The counter index in equation (5) was considered large enough that the change in its function value was less than or equal to 10^{-6}. Noticeably, the D_e value of each component changes throughout the extraction due to variation in the composition, and the reported D_e values in this table are just average values. According to Table 4, the diffusion coefficients of the components changed from 0.059×10^{-13} m^2/s for stearic acid to 1.187×10^{-13} m^2/s for linoleic acid; however, the diffusion coefficient of the extract is higher due to the availability of other undetermined components in the extract. The highest extraction yield was obtained for linoleic acid (26.8 mg/g raw material), followed by genipin (16.9 mg/g raw material). The maximum extraction yield for linolenic acid and stearic acid were of the same magnitude, i.e., 4.9 and 4.3 mg/g raw material, respectively, while palmitic acid had the lowest extraction yield of 3.0 mg/g raw material.

Table 4: The numerical values of the adjustable parameters of the HSD model.

Component	$Yield_{max}$ (mg/g raw material)	D_e (m^2/s) $\times 10^{13}$
Extract	60.29	1.891
Palmitic acid	3.02	0.675
Stearic acid	4.25	0.059
Linoleic acid	26.83	1.187
Linolenic acid	4.91	1.028
Genipin	16.88	0.558

To evaluate the prediction capability of the HSD model graphically, comparisons between the models and experimental data are illustrated in Figure 3. The data points in this figure are the average of two runs, and the error bars indicate the corresponding standard deviation. The dashed lines of the HSD model, as shown in this figure, pass precisely through the experimental data points. The HSD model has better agreement with the experimental data at higher extraction times. This difference in accuracy can be attributed to the fact that the diffusion mass transfer mechanism, which was employed in this study, is more reliable for the later stages of extraction. However, the main mechanism in the early stages of extraction is the convection mass transfer between solid and supercritical fluid, which was ignored in the HSD model.

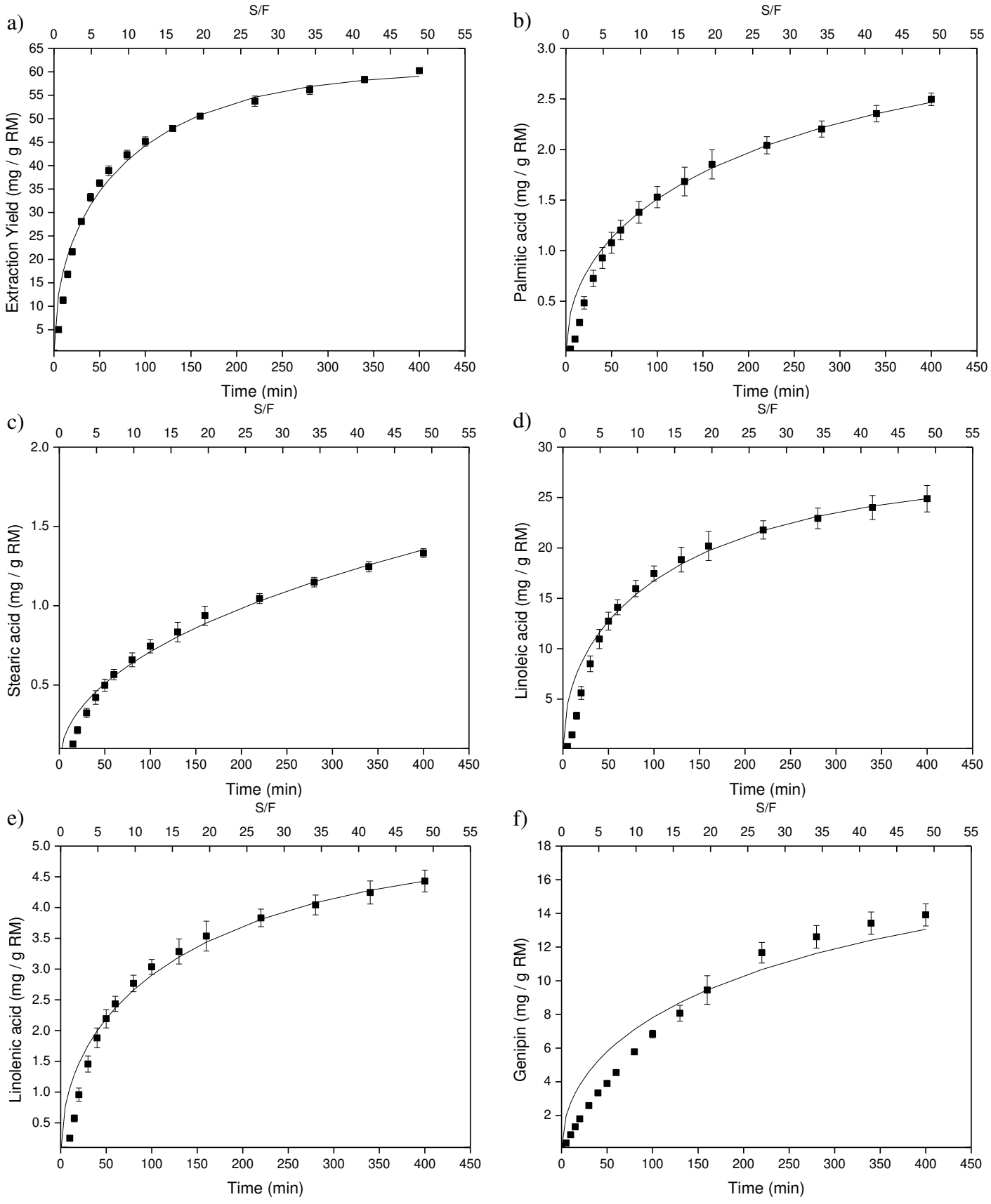


Figure 3. Comparison between the HSD model (dashed lines) and the experimental data (data points). a) Extraction yield; b) Palmitic acid; c) Stearic acid; d) Linoleic acid; e) Linolenic acid; f) Genipin. RM: raw material. All results are expressed in a dry base.

3.4 Process integration: obtaining fatty acids and genipin

Process integration for obtaining fatty acids and genipin-rich extracts was performed in two steps. In the first step, SFE was carried out under the optimized conditions selected in the first part of this study (Section 2.4), namely, temperature, pressure, S/F and flow rate of 333 K, 30 MPa, 16 g CO_2/g genipap, and 2.5 g/min, respectively. In the second step, the defatted raw material was submitted to LPSE using water as solvent for the recovery of genipin. According to a previous study by Náthia-Neves, Vardanega and Meireles [8], the optimum conditions for the LPSE process are temperature, pressure, S/F, and solvent flow rate of 313 K, 0.1 MPa, 20 g water/g genipap and 2 g / min, respectively. Table 5 presents the results obtained in the integrated process. In the first step, a fatty acid-rich extract mainly composed of linoleic acid was obtained (10.9 ± 0.8 mg/g RM), which is an important unsaturated fatty acid that represents approximately 1% of the unripe genipap fruit. In the second step, LPSE allowed the recovery of 71 ± 6 mg / g RM of genipin (approximately 7% of unripe genipap). The results obtained in this study are in agreement with those reported in the literature regarding the genipin content of unripe genipap fruit (1 to 9%) [32, 50]. Náthia-Neves, Vardanega and Meireles [8] recovered 80 ± 6 mg genipin / g RM using only the LPSE method. Thus, it can be concluded that the use of a defatted raw material does not alter the yield of genipin and that performing the SFE process prior to the extraction of genipin with water does not promote degradation of this compound.

The integration of the SFE process with other techniques to obtain different products from the same raw material has already proved to be successful. For example, Moraes, Zabot and Meireles [13] studied the integration of SFE-LPSE to obtain bixin and tocotrienol-rich oil from annatto seeds; Osorio-Tobón, Carvalho, Rostagno, Petenate and Meireles [51] studied the integration of SFE-PLE-SAS to obtain volatile oil and powdered curcuminoid-rich extract from turmeric; and Cardenas-Toro, Forster-Carneiro, Rostagno,

Petenate, Maugeri Filho and Meireles [14] studied the integration of SFE-subcritical water hydrolysis to obtain carotenoids and sugars from pressed palm fiber.

Table 5. Integrated process to obtain fatty acid (SFE process) and genipin (LPSE process).

	SFE Process T = 333 K and P = 30 MPa	LPSE Process T = 313 K and P = 0.1 MPa
Fatty acid (mg/g RM)		
Palmitic	1.6 ± 0.1	-
Stearic	0.9 ± 0.1	-
Linoleic	10.9 ± 0.8	-
Linolenic	2.5 ± 0.2	-
Genipin content (mg /g RM)	2.2 ± 0.2	71± 6

RM: ram material.

4. Conclusion

The results of this study show that it is possible to obtain an extract rich in fatty acids, consisting mainly of linoleic acid (76%), from unripe genipap fruit. The optimum conditions for the fatty acid extraction were temperature of 333 K and pressure of 30 MP, which extracted a total fatty acid content of 16.6 mg/g of unripe genipap. According to the HSD model, the highest extraction yields for the involved components ranged from 26.83 mg/g raw material for linoleic acid to 3.02 mg/g raw material for palmitic acid. The model also predicted that the diffusion coefficient for the components were between 0.059×10^{-13} m^2/s and 1.187×10^{-13} m^2/s. Furthermore, the integration of SFE with LPSE allowed obtaining a fatty acid-rich extract and a genipin-rich extract, which are products of great industrial interest.

Acknowledgments

G. Náthia-Neves thanks CAPES - Coordination of Superior Level Staff Improvement - Brazil (Finance Code 001) for a Ph.D. assistantship. T. Hatami thanks CAPES (Finance Code 001) for a postdoctoral assistantship, and M. A. A. Meireles thanks CNPq for a productivity grant (302423/2015-0).

References

[1] M. Herrero, E. Ibáñez, Green processes and sustainability: An overview on the extraction of high added-value products from seaweeds and microalgae, The Journal of Supercritical Fluids, 96 (2015) 211-216.
[2] J. Moncada B, V. Aristizábal M, C.A. Cardona A, Design strategies for sustainable biorefineries, Biochemical Engineering Journal, (2016).
[3] F. Fava, G. Totaro, L. Diels, M. Reis, J. Duarte, O.B. Carioca, H.M. Poggi-Varaldo, B.S. Ferreira, Biowaste biorefinery in Europe: opportunities and research & development needs, New biotechnology, 32 (2015) 100-108.
[4] L. Santos-Zea, J.A. Gutiérrez-Uribe, J. Benedito, Effect of ultrasound intensification on the supercritical fluid extraction of phytochemicals from Agave salmiana bagasse, The Journal of Supercritical Fluids, 144 (2019) 98-107.
[5] L.J. Rovetto, N.V. Aieta, Supercritical carbon dioxide extraction of cannabinoids from Cannabis sativa L, The Journal of Supercritical Fluids, 129 (2017) 16-27.
[6] J.P. Coelho, R.M. Filipe, M.P. Robalo, R.P. Stateva, Recovering value from organic waste materials: Supercritical fluid extraction of oil from industrial grape seeds, The Journal of Supercritical Fluids, 141 (2018) 68-77.
[7] E. Alonso, The role of supercritical fluids in the fractionation pretreatments of a wheat bran-based biorefinery, The Journal of Supercritical Fluids, 133 (2018) 603-614.
[8] G. Náthia-Neves, R. Vardanega, M.A.A. Meireles, Extraction of natural blue colorant from Genipa americana L. using green technologies: Techno-economic evaluation, Food and Bioproducts Processing, 114 (2019) 132-143.
[9] A. Demirbas, Biorefineries: Current activities and future developments, Energy Conversion and Management, 50 (2009) 2782-2801.
[10] J.A. Garcia-Nunez, D.T. Rodriguez, C.A. Fontanilla, N.E. Ramirez, E.E. Silva Lora, C.S. Frear, C. Stockle, J. Amonette, M. Garcia-Perez, Evaluation of alternatives for the evolution of palm oil mills into biorefineries, Biomass and Bioenergy, (2016).
[11] M. Boukroufa, C. Boutekedjiret, L. Petigny, N. Rakotomanomana, F. Chemat, Bio-refinery of orange peels waste: a new concept based on integrated green and solvent free extraction processes using ultrasound and microwave techniques to obtain essential oil, polyphenols and pectin, Ultrasonics sonochemistry, 24 (2015) 72-79.
[12] J.R. Bastidas-Oyanedel, C. Fang, S. Almardeai, U. Javid, A. Yousuf, J.E. Schmidt, Waste biorefinery in arid/semi-arid regions, Bioresource technology, 215 (2016) 21-28.
[13] M.N. Moraes, G.L. Zabot, M.A.A. Meireles, Extraction of tocotrienols from annatto seeds by a pseudo continuously operated SFE process integrated with low-pressure solvent extraction for bixin production, The Journal of Supercritical Fluids, 96 (2015) 262-271.
[14] F.P. Cardenas-Toro, T. Forster-Carneiro, M.A. Rostagno, A.J. Petenate, F. Maugeri Filho, M.A.A. Meireles, Integrated supercritical fluid extraction and subcritical water hydrolysis for the recovery of bioactive compounds from pressed palm fiber, The Journal of Supercritical Fluids, 93 (2014) 42-48.
[15] J.F. Osorio-Tobón, P.I.N. Carvalho, M.A. Rostagno, M.A.A. Meireles, Process integration for turmeric products extraction using supercritical fluids and pressurized liquids: Economic evaluation, Food and Bioproducts Processing, 98 (2016) 227-235.
[16] G.L. Zabot, M.A.A. Meireles, On-line process for pressurized ethanol extraction of onion peels extract and particle formation using supercritical antisolvent, The Journal of Supercritical Fluids, 110 (2016) 230-239.
[17] A.-j. Hu, S. Zhao, H. Liang, T.-q. Qiu, G. Chen, Ultrasound assisted supercritical fluid extraction of oil and coixenolide from adlay seed, Ultrasonics sonochemistry, 14 (2007) 219-224.
[18] G. Náthia-Neves, M.A.A. Meireles, Genipap: A New Perspective on Natural Colorants for the Food Industry, Food and Public Health, 8 (2018) 21-33.
[19] M.Á. Rincón-Cervera, V. González-Barriga, R. Valenzuela, S. López-Arana, J. Romero, A. Valenzuela, Profile and distribution of fatty acids in edible parts of commonly consumed marine fishes in Chile, Food chemistry, 274 (2019) 123-129.
[20] E. Scorletti, C.D. Byrne, Omega-3 fatty acids and non-alcoholic fatty liver disease: Evidence of efficacy and mechanism of action, Molecular Aspects of Medicine, 64 (2018) 135-146.

[21] D.F. Tirado, M.J. Tenorio, A. Cabañas, L. Calvo, Prediction of the best cosolvents to solubilise fatty acids in supercritical CO2 using the Hansen solubility theory, Chemical Engineering Science, 190 (2018) 14-20.
[22] S. Piskernik, R. Vidrih, L. Demšar, D. Koron, M. Rogelj, T.P. Žontar, Fatty acid profiles of seeds from different Ribes species, LWT, 98 (2018) 424-427.
[23] A. Standard, Method of determining and expressing particle size of chopped forage material by screening, St. Joseph. MI: ASAE, (1998).
[24] AOAC, Official methods of analysis of the Association of Official Analytical Chemistry, 16th ed., AOAC International, Gaithersburg, USA (1997), 1997.
[25] J. Crank, The mathematics of diffusion, Oxford university press, 1979.
[26] B. Ebrahimi, T. Hatami, J.H. Vera, Use of a hybrid optimization method to reduce vapor–liquid equilibrium data of maverick systems: The case of carbon dioxide with 2-methoxyethanol and 2-ethoxyethanol using cubic equations of state, Fluid Phase Equilibria, 338 (2013) 46-53.
[27] J. Joseph, R. Ackman, Capillary column gas chromatogrphic method for analysis of encapsulated fish oils and fish oil ethyl esters: collaborative study, Journal of AOAC International, (1992).
[28] M.M. Pollierer, J. Dyckmans, S. Scheu, D. Haubert, K. Treseder, Carbon flux through fungi and bacteria into the forest soil animal food web as indicated by compound-specific 13C fatty acid analysis, Functional Ecology, 26 (2012) 978-990.
[29] G. Náthia-Neves, G. Nogueira, R. Vardanega, M.A.A. Meireles, Identification and quantification of genipin and geniposide from Genipa americana L. by HPLC-DAD using a fused-core column, Food Science and Technology (Campinas), (2018).
[30] N.T. DUNFORD, F. TEMELLI, Extraction Conditions and Moisture Content of Canola Flakes as Related to Lipid Composition of Supercritical CO2 Extracts, Journal of Food Science, 62 (1997) 155-159.
[31] R.G.C.L. Porto, B.V.S. Cardoso, N.V.d.A. Barros, E.M.F. Cunha, M.A.d.M. Araújo, R.S.d.R. Moreira-Araújo, Chemical Composition and Antioxidant Activity of Genipa Americana L. (Jenipapo) of the Brazilian Cerrado, Journal of Agriculture and Environmental Sciences, 3 (2014).
[32] G. Náthia-Neves, A.G. Tarone, M.M. Tosi, M.R. Maróstica Júnior, M.A.A. Meireles, Extraction of bioactive compounds from genipap (Genipa americana L.) by pressurized ethanol: Iridoids, phenolic content and antioxidant activity, Food Research International, 102 (2017) 595-604.
[33] R.W. Figueiredo, G.A. Maia, L.F.F. Holanda, J.C. Monteiro, Características físicas e químicas do jenipapo, Pesquisa Agropecuária Brasileira, 21 (1986).
[34] A.d.S. Bentes, H.A.L. de Souza, J. Amaya-Farfan, A.S. Lopes, L.J.G. de Faria, Influence of the composition of unripe genipap (Genipa americana L.) fruit on the formation of blue pigment, J Food Sci Technol, 52 (2015) 3919-3924.
[35] P. dos Santos, A.C. de Aguiar, J. Viganó, J.S. Boeing, J.V. Visentainer, J. Martínez, Supercritical CO2 extraction of cumbaru oil (Dipteryx alata Vogel) assisted by ultrasound: Global yield, kinetics and fatty acid composition, The Journal of Supercritical Fluids, 107 (2016) 75-83.
[36] J. Viganó, J.P. Coutinho, D.S. Souza, N.A.F. Baroni, H.T. Godoy, J.A. Macedo, J. Martínez, Exploring the selectivity of supercritical CO2 to obtain nonpolar fractions of passion fruit bagasse extracts, The Journal of Supercritical Fluids, 110 (2016) 1-10.
[37] A. Hurtado-Benavides, D. Dorado A, A.d.P. Sánchez-Camargo, Study of the fatty acid profile and the aroma composition of oil obtained from roasted Colombian coffee beans by supercritical fluid extraction, The Journal of Supercritical Fluids, 113 (2016) 44-52.
[38] S.A.V. de Melo, G.M. Costa, A.C. Viana, F.L. Pessoa, Computation of Crossover Pressure for Synthesis of Supercritical Fluid Separation Systems, in: Computer Aided Chemical Engineering, Elsevier, 2009, pp. 399-404.
[39] N. Mezzomo, B.R. Mileo, M.T. Friedrich, J. Martinez, S.R. Ferreira, Supercritical fluid extraction of peach (Prunus persica) almond oil: process yield and extract composition, Bioresource technology, 101 (2010) 5622-5632.
[40] G. Gustinelli, L. Eliasson, C. Svelander, M. Alminger, L. Ahrné, Supercritical CO2 extraction of bilberry (Vaccinium myrtillus L.) seed oil: Fatty acid composition and antioxidant activity, The Journal of Supercritical Fluids, 135 (2018) 91-97.
[41] B. Purschke, T. Stegmann, M. Schreiner, H. Jäger, Pilot-scale supercritical CO2extraction of edible insect oil fromTenebrio molitorL. larvae - Influence of extraction conditions on kinetics,

defatting performance and compositional properties, European Journal of Lipid Science and Technology, 119 (2017) 1600134.
[42] O. Benito-Román, M. Rodríguez-Perrino, M.T. Sanz, R. Melgosa, S. Beltrán, Supercritical carbon dioxide extraction of quinoa oil: Study of the influence of process parameters on the extraction yield and oil quality, The Journal of Supercritical Fluids, 139 (2018) 62-71.
[43] P. dos Santos, A.C. de Aguiar, J. Viganó, J.S. Boeing, J.V. Visentainer, J. Martínez, Supercritical CO 2 extraction of cumbaru oil (Dipteryx alata Vogel) assisted by ultrasound: Global yield, kinetics and fatty acid composition, The Journal of Supercritical Fluids, 107 (2016) 75-83.
[44] S. Yehuda, S. Rabinovitz, R. L. Carasso, D. I. Mostofsky, The role of polyunsaturated fatty acids in restoring the aging neuronal membrane, Neurobiology of Aging, 23 (2002) 843-853.
[45] K.A. Youdim, A. Martin, J.A. Joseph, Essential fatty acids and the brain: possible health implications, International Journal of Developmental Neuroscience, 18 (2000) 383-399.
[46] S. Pepe, Effect of dietary polyunsaturated fatty acids on age-related changes in cardiac mitochondrial membranes, Experimental Gerontology, 40 (2005) 369-376.
[47] P.A.d. Costa, C.A. Ballus, J. Teixeira Filho, H.T. Godoy, Fatty acids profile of pulp and nuts of Brazilian fruits, Food Science and Technology, 31 (2011) 950-954.
[48] M.A.A. Meireles, Extraction of bioactive compounds from Latin American plants, Supercritical fluid extraction of nutraceuticals and bioactive compounds, (2008) 243-274.
[49] J.F. Soares, G.L. Zabot, M.V. Tres, F.C. Lunelli, V.M. Rodrigues, M.T. Friedrich, C.A. Pazinatto, D. Bilibio, M.A. Mazutti, N. Carniel, W.L. Priamo, Supercritical CO2 extraction of black poplar (Populus nigra L.) extract: Experimental data and fitting of kinetic parameters, The Journal of Supercritical Fluids, 117 (2016) 270-278.
[50] A.M. Ramos-de-la-Pena, J.C. Montanez, L. Reyes-Vega Mde, J.C. Contreras-Esquivel, Temperature model for process impact non-uniformity in genipin recovery by high pressure processing, Food chemistry, 187 (2015) 444-450.
[51] F.J. Osorio-Tobón, P.I.N. Carvalho, M.A. Rostagno, A.J. Petenate, M.A.A. Meireles, Extraction of curcuminoids from deflavored turmeric (Curcuma longa L.) using pressurized liquids: Process integration and economic evaluation, The Journal of Supercritical Fluids, 95 (2014) 167-174.

- *CHAPTER 7* -

GENERAL DISCUSSION

7 GENERAL DISCUSSION

The search for a healthier diet has been a worldwide trend, thus increasing the consumption of natural additives. In view of the demand of modern consumers and the limitations imposed by the current legislation on the use of synthetic additives, industries have opted for the increasing exploitation of natural colorants. In this sense, this thesis was developed aiming the use of emerging technologies to obtain a natural blue colorant of great interest for the food industry.

According to the literature review presented in **Chapter 2**, there are few natural pigments of blue color. The main ones come from some anthocyanins (obtained from cabbage and purple sweet potato, for instance), from phicocianin (obtained from *Spirulina platensis*) and from genipin (obtained directly from genipap or from β-glycoside hydrolysis of the geniposide). This bibliographical research focused on the use of genipap to obtain the blue pigment was useful to know about the physicochemical characteristics of this fruit as well as to understand the main factors that influence the mechanism of the blue color formation, such as the ripeness stage of the fruit, pH and protein content. According to this review only unripe fruits present the genipin iridoid, which is the compound responsible for the formation of blue color. Genipin is mainly present in unripe genipap, and besides coloring, this compound plays beneficial roles in the human body as antioxidant, anti-inflammatory and anticancer. This review also allowed obtaining information from studies involving the extraction of genipin in which it could be verified that the use of emerging technologies like pressurized liquids and supercritical fluids were little or never studied for the recovery of this compound.

Most of the methods available in the literature for the quantification of iridoids, such as genipin and geniposide, involve analytical runs with long running times. Therefore, the method developed and validated in **Chapter 3** is appropriate for the identification and quantification of these compounds found in genipap. The total HPLC run time was only 13 min, and the method was efficient in terms of resolution, selectivity and symmetry of the peaks.

Despite the information obtained in Chapter 2 that the endocarp is the part with the highest content of genipin, this part represents only 12% of the fruit, which could turn its use unfeasible at industrial scale. Therefore, the extraction of different parts of the unripe genipap (whole fruit, peel, mesocarp, endocarp, seeds and endocarp + seeds) were studied in **Chapter 4** using pressurized ethanol. Actually the endocarp stood out as the genipin richest part. However, the extraction yield using the whole fruit was greater than using only the endocarp and the content of genipin found in the whole fruit was very similar to that observed in the

endocarp. This result encourages the use of the whole fruit for the genipin recovery instead of the endocarp only, because it eliminates steps of separation of the parts and consequently can reduce in the costs of the process. In addition to the genipin content, geniposide content, a precursor of genipin that is present mainly in mesocarp and genipap peel, the phenolic content and the antioxidant activity of the extracts obtained were analyzed. All the studied parts showed phenolic compounds and antioxidant activity. However, the mesocarp presented higher phenolic content and antioxidant activity as measured by the DPPH method and the endocarp presented higher antioxidant activity measured by FRAP.

Although the whole fruit was a good source of genipin, the obtained extract showed green coloration. This can be explained by the presence of chlorophyll in the genipap peel that contributes to the formation of green color. As this research is focused on obtaining the blue color, tests were performed using whole fruit without the peel demonstrating satisfactory preliminary results, since the content of genipin was not altered by the removal of the peel and the obtained extract showed blue coloration. Thus, the optimization of genipin extraction using genipap without the peel was studied in **Chapter 5**. The variables studied in this chapter were solvent (water and ethanol), temperature (40, 50 and 60 °C) and pressure (0.1, 2, 5, and 8 MPa) and the results demonstrated that water is the best solvent for genipin recovery. Temperature and pressure did not exert significant influence (ANOVA, $\alpha = 0.05$) on genipin recovery. Therefore, considering energy costs the genipin extraction can be performed at 40 °C and at atmospheric pressure. The kinetics of two processes were also studied: *i)* using water at 40 °C and ambient pressure (LPSE) and *ii)* LPSE assisted by cold pressing (Press + LPSE). Genipine recovery of 8.3% was achieved in 22 min in the LPSE process while the Press + LPSE process allowed obtaining a similar amount of genipin of 7.7% in a shorter time (5.8 min).

The data obtained with the kinetic experiments were used as input data for the economic evaluation of the both LPSE and Press + LPSE processes. The simulations were performed in the SuperPro Designer® 8.5 software and scenarios considering different raw material acquisition prices (US$ 1.42 / kg and US$ 7.89 / kg), production scales (10, 50 and 100L) and genipin sales prices (US$ 50.00 to 250.00 / kg) were compared. From the economic point of view, both processes are applicable at industrial scales. The cost of acquisition of raw material and the sale prices greatly influenced the economic viability of the process. Lower costs of manufacturing (COM) were obtained for the larger scale production scenarios, *i.e.*, extraction plants with two 100 L extractors. The extract productivity obtained in the Press+LPSE process was 1.3 times higher than that in the LPSE process. The higher

productivity of the Press+LPSE process is related to its shorter process time, which, for instance, allows more batches per year than the LPSE process. Regarding the items that made up the COM, fixed capital invested (facilities) was the main component of COM when the cost of acquiring the raw material was US$ 1.42/kg. When the raw material cost was increased to US$ 7.89/kg, the raw material had a majority share in the COM of both processes. The sensitivity study showed that the scale of production and the marketing price of the products play an important role in the sustainability of the extraction plant. For genipin processing, the best option, among the scenarios studied in both processes, was the one containing a plant with two 100 L extractors, commercializing the extracts at a price of US$ 100.00/kg and US$ 200.00/kg, considering the cost with the raw material of US$ 1.42/kg and US$ 7.89/kg, respectively. This scenario presented better gross margin, return on investment, net present value and internal rate of return.

Although the main bioactive compound of unripe genipap fruit is genipin, this fruit is also a source of non-polar compounds such as fatty acids. The optimization of extraction from unripe genipap fruit using supercritical CO_2 was investigated in **Chapter 6**. The effects of temperature and pressure were evaluated and 60 °C and 30 MPa were selected as the best conditions to recover the genipap extract (yield of 4.6 ± 0.1%). The fatty acids present in this extract were linoleic acid (276 ± 20 mg/g extract) followed by linolenic acid (48 ± 4 mg/g extract), palmitic acid (24 ± 2 mg/g extract) and stearic acid (14 ± 2 mg/g extract). The kinetics results showed that in 450 minutes of process a yield of 6% was obtained and that the highest fatty acid yield was for linoleic acid (2.5 mg/g of genipap). However, in order to reduce the operational time and costs, it is suggested that the extraction of the unripe genipap extract can be finished at 112 minutes (S/F = 16), since from this time the increase on the yield is slow and, therefore, it would be more advantageous to start a new batch than to continue with the same extraction. The kinetic data were adjustable by the HSD model. According to this model, the highest extraction yield was obtained for linoleic acid, 26.8 mg/g genipap, followed by genipin, 16.9 mg/g genipap. The maximum extraction yields for linolenic acid and stearic acid were of the same magnitude, 4.9 and 4.3 mg/g genipap respectively, while palmitic acid had the lowest extraction yield, 3.0 mg/g genipap. It is worth mentioning that even having polar characteristics genipin was also found in the extract obtained by SFE which can be explained by the prolonged effect of the high pressure and the extraction temperature which may have promoted cell disruption and facilitated the mass transfer of other solutes to the solvent. In order to make the most of the biomass, the integration of the SFE and LPSE processes was studied,

where in the first stage an oil rich in fatty acids was obtained and the biomass resulting from this process was subjected to extraction with water at low pressure to obtain the blue colorant. In this integrated process it was possible to obtain almost 11 mg of linoleic/g of genipap and 71 mg of genipin/g of genipap.

The extracts obtained in this study presented characteristics that meet the market trends of products destined to food, pharmacological and cosmetic applications, and could thus become ingredients of products of these industrial lines.

- *CHAPTER 8* -

CONCLUSION AND SUGGESTIONS FOR FUTURE WORK

8.1 CONCLUSION

The processes developed in this work show to be technically efficient in obtaining an extract that can be used as a natural blue colorant. The analytical method developed and validated in Chapter 3 allowed the identification and quantification of genipin and geniposide iridoids present in genipap extracts in a short analysis time (13 min) and was efficient in terms of resolution, selectivity and symmetry of the peaks, which make it feasible to be used by the industry for analysis of these compounds.

The extraction process using pressurized ethanol (Chapter 4) was effective for the genipin extraction, geniposide and total phenolics in different parts of the unripe genipap fruit. The obtained extracts also presented antioxidant activity measured by the DPPH and FRAP methods. Based on the experiences obtained in this study, was conclude that:

- The studied pressures (2, 12 and 20 bar) did not interfere with genipin recovery while the temperatures studied (50 and 80 ° C) had a statistically significant influence on the recovery of this compound;
- The endocarp and the whole fruit had the highest content of genipin. While the mesocarp had higher levels of geniposide;
- The mesocarp was also detached because it presented higher content of TPC and DPPH, while the endocarp presented higher values of FRAP;
- Depending on the compound of interest, it is possible to use different parts of the genipap. Despite the high amount of genipin obtained from the whole fruit, its use to recover the blue colorant is conditioned by the need for other purification steps.

In Chapter 5 the extraction with pressurized liquids, the extraction at low pressure and the extraction at low pressure assisted by cold pressing were studied. The processes that stood out in this chapter in terms of overall yield and genipin recovery were economically evaluated. From the development of this study it was concluded that:

- The studied pressures and temperatures did not have significant influence on overall yield and genipin recovery. Therefore, from an energetic point of view, the genipin extraction process can be carried out at low pressures and temperatures;
- The LPSE process allowed a recovery of 93% genipin in 22 minutes;
- The Press+LPSE process allowed a recovery of 90% of genipin less than 6 minutes;
- The LPSE and Press + LPSE extraction processes were economically feasible when applied at the studied production scales (10, 50 and 100 L);

- The increased scale has raised productivity and reduced manufacturing cost in both processes;
- The sensitivity study showed that the scale of production, the price of the raw material acquisition and the sale price of the extract play an important role in the economic viability of an extraction plant.

In Chapter 6 the extraction of the unripe genipap fruit with supercritical carbon dioxide was studied. The extract obtained in this study presented higher content of linolenic acid, an essential fatty acid. This study allowed the following conclusions:

- The pressure and the interaction between pressure and temperature significantly influenced X0 in the studied range;
- As there was no difference in the global yield and composition of the fatty acids extracted at 300 and 350 bar at 60 °C, it is suggested that extraction from unripe genipap can be carried out at pressure of 300 bar and temperature of 60 °C;
- No significant differences in the fatty acid profiles were observed, that is, in all the extracts were found the same fatty acids;
- The extraction of the unripe genipap oil can be interrupted at the 112 minutes (S/F = 16);
- The recovery of genipin in the oil extract obtained by SFE was 14 mg/g of genipap;
- According to the HSD model, the highest extraction yields for the involved components ranged from 26.83 mg/g raw material for linoleic acid to 3.02 mg/g raw material for palmitic acid;
- The integration of SFE with LPSE allowed obtaining fatty acids-rich extract and genipin-rich extract which are products of great industrial interest.

8.2 SUGGESTIONS FOR FUTURE WORKS

After performing the activities detailed in the thesis and with the information obtained, the suggestions listed below aim to stimulate continued research on the subject:

- To study the extraction of the genipap peel, since it was not used for the extraction of the genipin;
- To quantify the phenolic compounds present in extracts rich in genipin;
- To study the extraction of genipin from unripe genipap *in natura* via ultrasound;

- To analyze the extract obtained by SFE in terms of phytosteroids, tocopherol and volatiles;
- To study the chemical composition and ways to reuse waste from the extraction, such as biofilm production or by subjecting them to hydrolysis for energy conversion;
- To evaluate the toxicity of the extracts obtained;
- To study possible extracts applications through cell and animal assays;
- To perform the scale up of the genipin extraction process.

MEMORY OF THE PERIOD OF DOCTORATE

MEMORY OF THE PERIOD OF DOCTORATE

Grazielle Náthia Neves joined the PhD program in Food Engineering (DEA/FEA/UNICAMP) in March 2016, receiving a PhD scholarship granted by CAPES from March 2016 to February 2020.

The courses taken over these three years were: TP 199 - Seminars (2 credits); TP 143 – Rheology (3 credits); TP 121 - Special Topics in Food Engineering – Food Materials Science (2 credits); TP 159 - Special Topics in Food Engineering - Academic Written (2 credits), TP 150 - Phase equilibria in food systems (2 credits). To achieve the number of credits required by the program, a course taken during the master's degree was validated: TP 150 – Special Topics in Food Engineering - Nanotechnology (2 credits).

In addition to the subjects studied, another 4 credits were fulfilled through participation in the Teaching Internship Program group C (PED C). In the second academic period of 2016 and 2018, she participated in the PED C with activities of partial support to teaching in the disciplines TA 731A - Unit Operations II and TA 331A - Thermodynamics both with a workload of 8 hours per week.

The doctoral student participated of the Congress of Scientific Initiation of Unicamp as evaluator of works enrolled in the Technological area in 2016 (Issue XXIV) and in 2017 (Issue XXV). In 2016 she participated in the IFST (Innovations in Food Science and Technology) congress, hold in Erding, Germany, presenting the work "Extraction of iridoids from genipap fruit applying pressurized liquid".

In January 2019, the doctoral student participated in an academic-cultural exchange - Sakura Exchange Program in Science held in Kumamoto - Japan. During this program she participated in The 49th IROAST Seminar with two presentations about Brazil and Unicamp and another about her doctoral research.

The activities related to this research project in addition to those carried out in cooperation with other researchers resulted 4 articles: one review article published in the journals *Food and Public Health*, and 3 experimental articles published in the journals *Food Science and Technology*, *Food Research International*, and *Food and Bioproducts Processing*. Also, during this period, a chapter was published in the *Handbook of Food Bioengineering*.

Articles:

Náthia-Neves, G., & Meireles, M. A. A. (2018). Genipap: A New Perspective on Natural Colorants for the Food Industry. Food and Public Health, 8(1), 21-33.

Náthia-Neves, G., Nogueira, G., Vardanega, R., & Meireles, M. A. A. (2018). Identification and quantification of genipin and geniposide from Genipa americana L. by HPLC-DAD using a fused-core column. Food Science and Technology (Campinas).

Náthia-Neves, G., Tarone, A. G., Tosi, M. M., Maróstica Júnior, M. R., & Meireles, M. A. A. (2017). Extraction of bioactive compounds from genipap (Genipa americana L.) by pressurized ethanol: Iridoids, phenolic content and antioxidant activity. Food Research International, 102(Supplement C), 595-604.

Náthia-Neves, G., Vardanega, R., & Meireles, M. A. A. (2019). Extraction of natural blue colorant from Genipa americana L. using green technologies: Techno-economic evaluation. Food and Bioproducts Processing, 114, 132-143.

Prado, M. J., Veggi, C.P., **Náthia-Neves, G.**, & Meireles, M. A. A. (2018). Extraction Methods for Obtaining Natural Blue Colorants. Current Analytical Chemistry 14, 1-28.

Abstracts:

Náthia-Neves, G.; Vardanega, R.; Meireles, M. A. A. . Extraction of iridoids from genipap fruit applying pressurized liquid. In: Innovations in Food Science and Technology, 2017, Erding. Innovations in Food Science and Technology, 2017. v. 1. p. 38-38.

Calzado, T. ; **Náthia-Neves, G.** ; Vardanega, R. ; Meireles, M. A. A. . Extração de corantes naturais de Genipa americana L.. In: XXV Congresso de Iniciação Científica da UNICAMP, 2017, Campinas. Extração de corantes naturais de Genipa americana L., 2017.

Book chapter:

Silva, E. K., Zabot, G. L., **Náthia-Neves, G.**, Nogueira, G. C., & Meireles, A. M. A. (2018). Chapter 12 - Process Engineering Applying Supercritical Technology for Obtaining Functional and Therapeutic Products Advances in Biotechnology for Food Industry (pp. 327-358): Academic Press.

Vardanega, R., **Náthia-Neves, G.**, Veggi, C.P., & Meireles, A. M. A. (2019). Chapter 3 Supercritical Fluid Processing and Extraction of Food in Green Food Processing Techniques. Academic Press.

GENERAL REFERENCES

GENERAL REFERENCES

BALAMURUGAN, M.; RAJESH, S.; MANOGARAN, E. 'Genipin' – The Natural Water Soluble Cross-linking Agent and Its Importance in the Modified Drug Delivery Systems: An Overview. Current Drug Delivery, v. 11, n. 1, p. 139-145, 2014. ISSN 1567-2018/1875-5704.

BENTES, A. S. et al. Influence of the composition of unripe genipap (Genipa americana L.) fruit on the formation of blue pigment. Journal of Food Science and Technology, v. 52, n. 6, p. 3919-3924, 2015/06/01 2015. ISSN 0022-1155.

BENTES, A. S.; MERCADANTE, A. Z. Influence of the Stage of Ripeness on the Composition of Iridoids and Phenolic Compounds in Genipap (Genipa americana L.). Journal of Agricultural and Food Chemistry, v. 62, n. 44, p. 10800-10808, 2014/11/05 2014. ISSN 0021-8561.

BRAUCH, J. E. et al. Jagua blue derived from Genipa americana L. fruit: A natural alternative to commonly used blue food colorants? Food Research International, v. 89, p. 391-398, 2016. ISSN 09639969.

CHEMAT, F. et al. Review of Green Food Processing techniques. Preservation, transformation, and extraction. Innovative Food Science & Emerging Technologies, v. 41, p. 357-377, 2017. ISSN 14668564.

CHEMAT, F.; VIAN, M. A.; CRAVOTTO, G. Green Extraction of Natural Products: Concept and Principles. International Journal of Molecular Sciences, v. 13, n. 7, p. 8615-8627, 07/11

ISSN 1422-0067.

DJERASSI, C.; GRAY, J. D.; KINCL, F. A. Naturally Occurring Oxygen Heterocyclics. IX.1Isolation and Characterization of Genipin2. The Journal of Organic Chemistry, v. 25, n. 12, p. 2174-2177, 1960. ISSN 0022-3263 1520-6904.

FIGUEIREDO, R. W. et al. Características físicas e químicas do jenipapo. Pesquisa Agropecuária Brasileira, v. 21, n. 4, 1986.

LEE, S.-W. et al. Colorimetric determination of amino acids using genipin from Gardenia jasminoides. Analytica Chimica Acta, v. 480, n. 2, p. 267-274, 3/24/ 2003. ISSN 0003-2670.

MARTINS, N. et al. Food colorants: Challenges, opportunities and current desires of agro-industries to ensure consumer expectations and regulatory practices. Trends in Food Science & Technology, v. 52, p. 1-15, 2016. ISSN 09242244.

MEIRELES, M. A. A. Extraction of bioactive compounds from Latin American plants. Supercritical fluid extraction of nutraceuticals and bioactive compounds, p. 243-274, 2008.

MUSTAFA, A.; TURNER, C. Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. Anal Chim Acta, v. 703, n. 1, p. 8-18, Oct 3 2011. ISSN 1873-4324 (Electronic) 0003-2670 (Linking).

NÁTHIA-NEVES, G.; MEIRELES, M. A. A. Genipap: A New Perspective on Natural Colorants for the Food Industry. Food and Public Health, v. 8, n. 1, p. 21-33, 2018. ISSN 2162-9412

NÁTHIA-NEVES, G. et al. Identification and quantification of genipin and geniposide from Genipa americana L. by HPLC-DAD using a fused-core column. Food Science and Technology (Campinas), 2018. ISSN 0101-2061.

NÁTHIA-NEVES, G. et al. Extraction of bioactive compounds from genipap (Genipa americana L.) by pressurized ethanol: Iridoids, phenolic content and antioxidant activity. Food Research International, v. 102, n. Supplement C, p. 595-604, 2017/12/01/ 2017. ISSN 0963-9969.

NÁTHIA-NEVES, G.; VARDANEGA, R.; MEIRELES, M. A. A. Extraction of natural blue colorant from Genipa americana L. using green technologies: Techno-economic evaluation. Food and Bioproducts Processing, v. 114, p. 132-143, 2019. ISSN 09603085.

OSORIO-TOBÓN, J. F.; MEIRELES, M. A. A. Recent Applications of Pressurized Fluid Extraction: Curcuminoids Extraction with Pressurized Liquids. Food and Public Health, v. 3, n. 6, p. 289-303, 2013. ISSN 2162-8440.

RAMOS-DE-LA-PENA, A. M. et al. Temperature model for process impact non-uniformity in genipin recovery by high pressure processing. Food Chem, v. 187, p. 444-50, Nov 15 2015. ISSN 0308-8146 (Print) 0308-8146 (Linking).

RAMOS-DE-LA-PEÑA, A. M. et al. Recovery of genipin from genipap fruit by high pressure processing. LWT - Food Science and Technology, v. 63, n. 2, p. 1347-1350, 2015. ISSN 00236438.

RAMOS-DE-LA-PENA, A. M. et al. Environmental friendly cold-mechanical/sonic enzymatic assisted extraction of genipin from genipap (Genipa americana). Ultrason Sonochem, v. 21, n. 1, p. 43-9, Jan 2014. ISSN 1873-2828 (Electronic) 1350-4177 (Linking).

RAMOS-DE-LA-PEÑA, A. M. et al. A review through recovery, purification and identification of genipin. Phytochemistry Reviews, v. 15, n. 1, p. 37-49, 2016. ISSN 1572-980X.

RAMOS-DE-LA-PEÑA, A. M. et al. Ultrafiltration for genipin recovery technologies after ultrasonic treatment of genipap fruit. Biocatalysis and Agricultural Biotechnology, v. 4, n. 1, p. 11-16, 2015. ISSN 18788181.

RENHE, I. R. T. et al. Obtenção de corante natural azul extraído de frutos de jenipapo. Pesquisa Agropecuária Brasileira, v. 44, p. 649-652, 2009. ISSN 0100-204X.

SOUZA, V. R. et al. Determination of bioactive compounds, antioxidant activity and chemical composition of Cerrado Brazilian fruits. Food Chemistry, v. 134, n. 1, p. 381-386, 2012. ISSN 03088146.

VARDANEGA, R. et al. Techno-economic evaluation of obtaining Brazilian ginseng extracts in potential production scenarios. Food and Bioproducts Processing, v. 101, p. 45-55, 2017. ISSN 09603085.

VAZQUEZ-ROIG, P.; PICÓ, Y. Pressurized liquid extraction of organic contaminants in environmental and food samples. TrAC Trends in Analytical Chemistry, v. 71, p. 55-64, 2015. ISSN 01659936.

VLYSIDIS, A. et al. A techno-economic analysis of biodiesel biorefineries: Assessment of integrated designs for the co-production of fuels and chemicals. Energy, v. 36, n. 8, p. 4671-4683, 2011. ISSN 03605442.

ZABOT, G. L.; MORAES, M. N.; MEIRELES, M. A. A. Supercritical Technology Applied to the Production of Bioactive Compounds: Research Studies Conducted at LASEFI from 2009 to 2013. Food and Public Health, v. 4, n. 2, p. 36-48, 2014. ISSN 2162-8440.

Appendix A

Appendix A comprises the experimental data for the calibration curves of the genipin and geniposide standards used in **Chapter 3.**

Table A.1: Data for the calibration curve for geniposide quantification in genipap.

C oncentration (µg/mL)	Area 1	Area 2	Area 3	Average
1000	9162877	9291908	9214298	9223028
625	5942693	5993390	5998088	5978057
312.5	3019730	3010650	3023071	3017817
156.25	1521774	1526304	1510057	1519378
104	1011681	1006878	1002074	1006878
52	519062	519419	504645	514375
26	274253	269670	254383	266102
6.5	85547	84397	69473	79806
1.63	42864	41107	23564	35845
0.4075	29603	33088	11973	24888
0.102	24499	26659	46351	32503

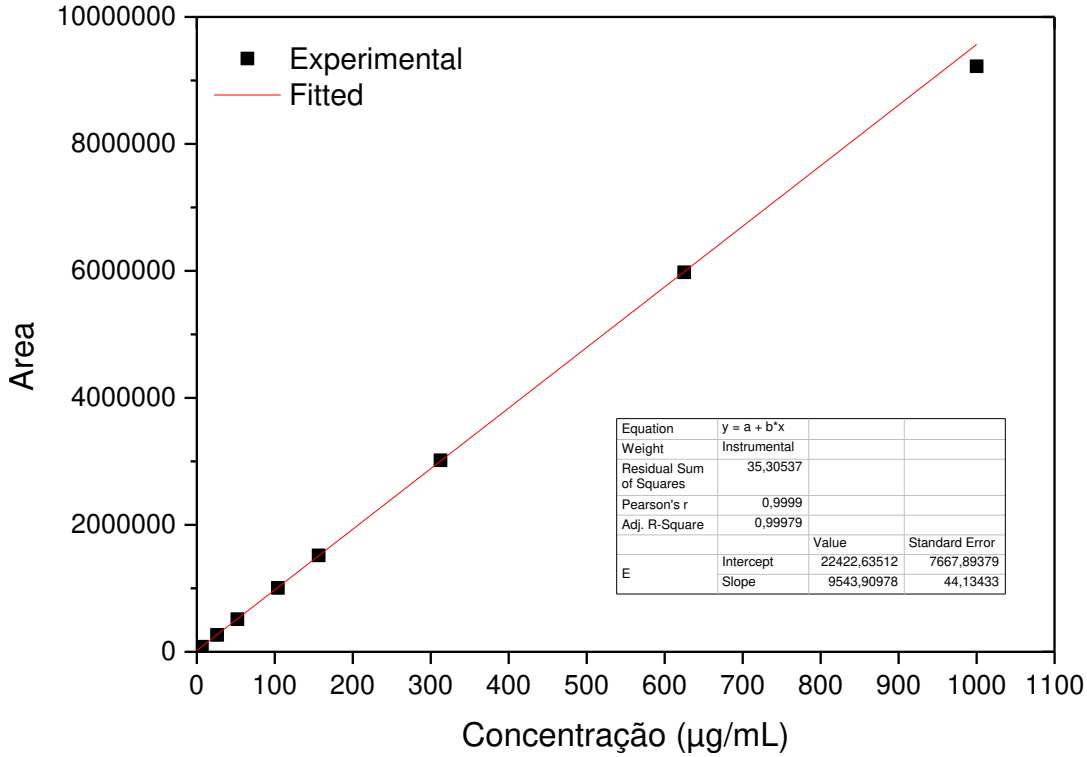


Figure A.1: Geniposide Calibration Curve.

Table A.2: Data for the calibration curve for genipin quantification in genipap.

C oncentration (µg/mL)	Area 1	Area 2	Area 3	Average
2500	21214554	20496730	20517273	20742852
1250	16365050	15824154	15988214	16059139
1000	13798721	13468480	13633601	13633601
625	9438301	9839476	9323316	9533698
312.5	4730273	4736731.5	4743190	4736732
156.25	2354529	2514070	2378633	2415744
104	1587414	1652591	1602775	1614260
52	1039614	857364	798404	898461
26	411103	433458	497437	447333
6.5	104758	114891	108960	109536
1.63	28757	42974	27129	32953
0.41	8164	26149	17156.5	17157
0.1	2335	11918	7126.5	7127

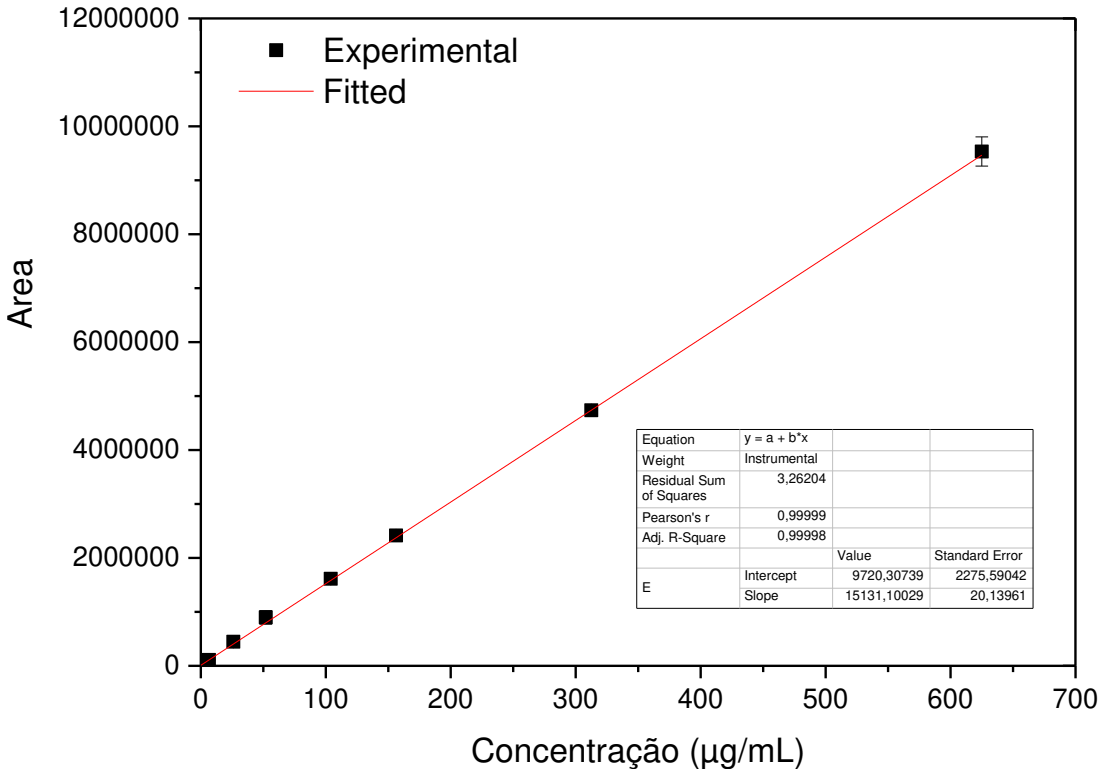


Figure A.2: Genipin Calibration Curve.

Appendix B

Appendix B contains supplemental information pertaining to **Chapter 4** which includes the analysis of variance (ANOVA) data generated for the experimental designs performed for the extraction of the different parts of the genipap using pressurized ethanol.

Table B.1: Analysis of variance (ANOVA) generated for the experimental planning of the parts of genipap.

General Linear Model: Yield (%) versus Parts of fruit, Temperature , ...

```
Factor            Type   Levels  Values
Parts of fruit    fixed       6  Endocarp + seeds, Seeds, Peel, Endocarp, Whole
                                 fruit, Mesocarp
Temperature (°C)  fixed       2  50, 80
Pressure (bar)    fixed       3  2, 12, 20

Analysis of Variance for Yield (%), using Adjusted SS for Tests

Source                            DF   Seq SS   Adj SS  Adj MS       F      P
Parts of fruit                     5  4021.07  4021.07  804.21  174.52  0.000
Temperature (°C)                   1    70.57    70.57   70.57   15.31  0.003
Pressure (bar)                     2    40.15    40.15   20.07    4.36  0.044
Parts of fruit*Temperature (°C)    5    12.52    12.52    2.50    0.54  0.740
Parts of fruit*Pressure (bar)     10    45.34    45.34    4.53    0.98  0.510
Temperature (°C)*Pressure (bar)    2     1.42     1.42    0.71    0.15  0.859
Error                             10    46.08    46.08    4.61
Total                             35  4237.15

S = 2.14666   R-Sq = 98.91%   R-Sq(adj) = 96.19%

Least Squares Means for Yield (%)

Parts of fruit              Mean  SE Mean
Endocarp + seeds           14.94   0.8764
Seeds                      16.19   0.8764
Peel                       22.23   0.8764
Endocarp                   25.52   0.8764
Whole fruit                36.62   0.8764
Mesocarp                   44.07   0.8764
Temperature
50                         25.19   0.5060
80                         27.99   0.5060
Pressure (bar)
 2                         27.20   0.6197
12                         27.47   0.6197
20                         25.11   0.6197
Parts of fruit*Temperature
Endocarp + seeds 50        12.63   1.2394
Endocarp + seeds 80        17.25   1.2394
Seeds        50            15.36   1.2394
Seeds        80            17.01   1.2394
Peel         50            21.50   1.2394
Peel         80            22.96   1.2394
Endocarp     50            24.43   1.2394
Endocarp     80            26.62   1.2394
Whole fruit  50            35.20   1.2394
Whole fruit  80            38.04   1.2394
Mesocarp     50            42.05   1.2394
Mesocarp     80            46.09   1.2394
Parts of fruit*Pressure (bar)
Endocarp + seeds  2        16.04   1.5179
Endocarp + seeds 12        14.50   1.5179
```

```
Endocarp + seeds 20        14.29   1.5179
Seeds         2            15.85   1.5179
Seeds        12            17.89   1.5179
Seeds        20            14.81   1.5179
Peel          2            23.05   1.5179
Peel         12            23.65   1.5179
Peel         20            19.98   1.5179
Endocarp      2            24.97   1.5179
Endocarp     12            28.50   1.5179
Endocarp     20            23.09   1.5179
Whole fruit   2            36.63   1.5179
Whole fruit  12            36.33   1.5179
Whole fruit  20            36.90   1.5179
Mesocarp      2            46.68   1.5179
Mesocarp     12            43.95   1.5179
Mesocarp     20            41.58   1.5179
Temperature *Pressure (bar)
50            2            25.91   0.8764
50           12            25.79   0.8764
50           20            23.88   0.8764
80            2            28.50   0.8764
80           12            29.15   0.8764
80           20            26.34   0.8764
```

General Linear Model: Geniposide (versus Parts of fruit, Temperature , ...

```
Factor            Type   Levels  Values
Parts of fruit    fixed       6  Endocarp + seeds, Seeds, Peel, Endocarp, Whole
                                 fruit, Mesocarp
Temperature (°C)  fixed       2  50, 80
Pressure (bar)    fixed       3  2, 12, 20

Analysis of Variance for Geniposide (mg / gRM), using Adjusted SS for Tests

Source                            DF    Seq SS    Adj SS   Adj MS       F      P
Parts of fruit                     5  14284.36  14284.36  2856.87  935.45  0.000
Temperature (°C)                   1     31.40     31.40    31.40   10.28  0.009
Pressure (bar)                     2     26.38     26.38    13.19    4.32  0.044
Parts of fruit*Temperature (°C)    5     49.62     49.62     9.92    3.25  0.053
Parts of fruit*Pressure (bar)     10     90.95     90.95     9.09    2.98  0.050
Temperature (°C)*Pressure (bar)    2      3.98      3.98     1.99    0.65  0.542
Error                             10     30.54     30.54     3.05
Total                             35  14517.23

S = 1.74757   R-Sq = 99.79%   R-Sq(adj) = 99.26%

Unusual Observations for Geniposide (mg / gRM)

     Geniposide
Obs  (mg / gRM)      Fit  SE Fit  Residual  St Resid
 31     59.3204  56.5893  1.4852    2.7311      2.97 R
 32     46.8691  48.8363  1.4852   -1.9672     -2.14 R
 34     47.5895  50.3205  1.4852   -2.7311     -2.97 R
 35     46.0890  44.1218  1.4852    1.9672      2.14 R

R denotes an observation with a large standardized residual.

Least Squares Means for Geniposide (mg / gRM)

Parts of fruit               Mean  SE Mean
Endocarp + seeds           0.2413   0.7134
Seeds                      0.0588   0.7134
Peel                      36.0366   0.7134
Endocarp                   0.1113   0.7134
Whole fruit                0.8529   0.7134
Mesocarp                  47.8517   0.7134
Temperature
```

```
50                          15.1261   0.4119
80                          13.2581   0.4119
Pressure (bar)
 2                          15.2653   0.5045
12                          14.1403   0.5045
20                          13.1706   0.5045
Parts of fruit*Temperature
Endocarp + seeds 50          0.1773   1.0090
Endocarp + seeds 80          0.3053   1.0090
Seeds         50             0.0643   1.0090
Seeds         80             0.0533   1.0090
Peel          50            38.4649   1.0090
Peel          80            33.6083   1.0090
Endocarp      50             0.0097   1.0090
Endocarp      80             0.2128   1.0090
Whole fruit   50             1.5135   1.0090
Whole fruit   80             0.1924   1.0090
Mesocarp      50            50.5268   1.0090
Mesocarp      80            45.1766   1.0090
Parts of fruit*Pressure (bar)
Endocarp + seeds  2          0.2174   1.2357
Endocarp + seeds 12          0.3393   1.2357
Endocarp + seeds 20          0.1673   1.2357
Seeds          2             0.0681   1.2357
Seeds         12             0.0846   1.2357
Seeds         20             0.0237   1.2357
Peel           2            37.1742   1.2357
Peel          12            37.0838   1.2357
Peel          20            33.8518   1.2357
Endocarp       2             0.1682   1.2357
Endocarp      12             0.0750   1.2357
Endocarp      20             0.0905   1.2357
Whole fruit    2             0.5092   1.2357
Whole fruit   12             0.7803   1.2357
Whole fruit   20             1.2692   1.2357
Mesocarp       2            53.4549   1.2357
Mesocarp      12            46.4790   1.2357
Mesocarp      20            43.6211   1.2357
Temperature *Pressure (bar)
50             2            16.6586   0.7134
50            12            14.7565   0.7134
50            20            13.9632   0.7134
80             2            13.8721   0.7134
80            12            13.5242   0.7134
80            20            12.3781   0.7134
```

General Linear Model: Genipin (mg versus Parts of fruit, Temperature , ...

```
Factor            Type   Levels  Values
Parts of fruit    fixed       6  Endocarp + seeds, Seeds, Peel, Endocarp, Whole
                                 fruit, Mesocarp
Temperature (°C)  fixed       2  50, 80
Pressure (bar)    fixed       3  2, 12, 20

Analysis of Variance for Genipin (mg / gRM), using Adjusted SS for Tests

Source                           DF   Seq SS   Adj SS   Adj MS      F      P
Parts of fruit                    5  7295.79  7295.79  1459.16  79.48  0.000
Temperature (°C)                  1   477.15   477.15   477.15  25.99  0.000
Pressure (bar)                    2    66.59    66.59    33.30   1.81  0.213
Parts of fruit*Temperature (°C)   5   510.81   510.81   102.16   5.57  0.010
Parts of fruit*Pressure (bar)    10   171.33   171.33    17.13   0.93  0.542
Temperature (°C)*Pressure (bar)   2     8.20     8.20     4.10   0.22  0.804
Error                            10   183.58   183.58    18.36
Total                            35  8713.45

S = 4.28459   R-Sq = 97.89%   R-Sq(adj) = 92.63%
```

Unusual Observations for Genipin (mg / gRM)

Obs	Genipin (mg / gRM)	Fit	SE Fit	Residual	St Resid
25	37.1658	42.8500	3.6412	-5.6841	-2.52 R
27	46.4867	41.0165	3.6412	5.4702	2.42 R
28	29.4479	23.7638	3.6412	5.6841	2.52 R
30	14.7696	20.2398	3.6412	-5.4702	-2.42 R

R denotes an observation with a large standardized residual.

Least Squares Means for Genipin (mg / gRM)

Parts of fruit	Mean	SE Mean
Endocarp + seeds	16.0966	1.749
Seeds	1.3389	1.749
Peel	6.6924	1.749
Endocarp	40.7222	1.749
Whole fruit	32.6164	1.749
Mesocarp	9.2113	1.749
Temperature		
50	21.4203	1.010
80	14.1390	1.010
Pressure (bar)		
2	17.9572	1.237
12	19.3495	1.237
20	16.0322	1.237
Parts of fruit*Temperature		
Endocarp + seeds 50	20.6460	2.474
Endocarp + seeds 80	11.5471	2.474
Seeds 50	1.7645	2.474
Seeds 80	0.9133	2.474
Peel 50	7.2592	2.474
Peel 80	6.1256	2.474
Endocarp 50	40.3218	2.474
Endocarp 80	41.1227	2.474
Whole fruit 50	42.3489	2.474
Whole fruit 80	22.8840	2.474
Mesocarp 50	16.1812	2.474
Mesocarp 80	2.2415	2.474
Parts of fruit*Pressure (bar)		
Endocarp + seeds 2	15.2052	3.030
Endocarp + seeds 12	18.0053	3.030
Endocarp + seeds 20	15.0793	3.030
Seeds 2	1.4030	3.030
Seeds 12	1.5898	3.030
Seeds 20	1.0240	3.030
Peel 2	6.8329	3.030
Peel 12	6.9474	3.030
Peel 20	6.2970	3.030
Endocarp 2	39.1622	3.030
Endocarp 12	48.2985	3.030
Endocarp 20	34.7058	3.030
Whole fruit 2	33.3069	3.030
Whole fruit 12	33.9143	3.030
Whole fruit 20	30.6282	3.030
Mesocarp 2	11.8333	3.030
Mesocarp 12	7.3416	3.030
Mesocarp 20	8.4591	3.030
Temperature *Pressure (bar)		
50 2	21.4085	1.749
50 12	22.5236	1.749
50 20	20.3287	1.749
80 2	14.5060	1.749
80 12	16.1754	1.749
80 20	11.7357	1.749

General Linear Model: TPC (mg GAE versus Parts of fruit, Temperature , ...

Factor	Type	Levels	Values
Parts of fruit	fixed	6	Endocarp + seeds, Seeds, Peel, Endocarp, Whole fruit, Mesocarp
Temperature (°C)	fixed	2	50, 80
Pressure (bar)	fixed	3	2, 12, 20

Analysis of Variance for TPC (mg GAE / gRM), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Parts of fruit	5	24525.8	24525.8	4905.2	137.78	0.000
Temperature (°C)	1	3460.3	3460.3	3460.3	97.20	0.000
Pressure (bar)	2	223.0	223.0	111.5	3.13	0.088
Parts of fruit*Temperature (°C)	5	1626.9	1626.9	325.4	9.14	0.002
Parts of fruit*Pressure (bar)	10	721.2	721.2	72.1	2.03	0.141
Temperature (°C)*Pressure (bar)	2	91.6	91.6	45.8	1.29	0.318
Error	10	356.0	356.0	35.6		
Total	35	31004.7				

S = 5.96667 R-Sq = 98.85% R-Sq(adj) = 95.98%

Least Squares Means for TPC (mg GAE / gRM)

Parts of fruit	Mean	SE Mean
Endocarp + seeds	18.08	2.436
Seeds	43.33	2.436
Peel	18.65	2.436
Endocarp	23.12	2.436
Whole fruit	61.22	2.436
Mesocarp	89.36	2.436
Temperature		
50	32.49	1.406
80	52.10	1.406
Pressure (bar)		
2	40.83	1.722
12	45.80	1.722
20	40.26	1.722
Parts of fruit*Temperature		
Endocarp + seeds 50	10.63	3.445
Endocarp + seeds 80	25.53	3.445
Seeds 50	35.33	3.445
Seeds 80	51.33	3.445
Peel 50	16.26	3.445
Peel 80	21.03	3.445
Endocarp 50	19.54	3.445
Endocarp 80	26.70	3.445
Whole fruit 50	44.77	3.445
Whole fruit 80	77.67	3.445
Mesocarp 50	68.41	3.445
Mesocarp 80	110.32	3.445
Parts of fruit*Pressure (bar)		
Endocarp + seeds 2	20.70	4.219
Endocarp + seeds 12	13.75	4.219
Endocarp + seeds 20	19.80	4.219
Seeds 2	30.50	4.219
Seeds 12	52.50	4.219
Seeds 20	47.00	4.219
Peel 2	18.85	4.219
Peel 12	20.55	4.219
Peel 20	16.55	4.219
Endocarp 2	24.00	4.219
Endocarp 12	28.90	4.219
Endocarp 20	16.45	4.219
Whole fruit 2	62.15	4.219
Whole fruit 12	63.00	4.219
Whole fruit 20	58.50	4.219
Mesocarp 2	88.77	4.219

Mesocarp	12	96.09	4.219
Mesocarp	20	83.23	4.219
Temperature *Pressure (bar)			
50	2	33.19	2.436
50	12	35.45	2.436
50	20	28.82	2.436
80	2	48.47	2.436
80	12	56.14	2.436
80	20	51.69	2.436

General Linear Model: FRAP (µmol T versus Parts of fruit, Temperature , ...

Factor	Type	Levels	Values
Parts of fruit	fixed	6	Endocarp + seeds, Seeds, Peel, Endocarp, Whole fruit, Mesocarp
Temperature (°C)	fixed	2	50, 80
Pressure (bar)	fixed	3	2, 12, 20

Analysis of Variance for FRAP (µmol TE / gRM), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Parts of fruit	5	63015	63015	12603	10.57	0.001
Temperature (°C)	1	48662	48662	48662	40.79	0.000
Pressure (bar)	2	3669	3669	1835	1.54	0.262
Parts of fruit*Temperature (°C)	5	7075	7075	1415	1.19	0.381
Parts of fruit*Pressure (bar)	10	11042	11042	1104	0.93	0.547
Temperature (°C)*Pressure (bar)	2	3448	3448	1724	1.45	0.281
Error	10	11929	11929	1193		
Total	35	148840				

S = 34.5383 R-Sq = 91.99% R-Sq(adj) = 71.95%

Unusual Observations for FRAP (µmol TE / gRM)

Obs	FRAP (µmol TE / gRM)	Fit	SE Fit	Residual	St Resid
20	107.000	145.832	29.352	-38.832	-2.13 R
21	137.000	84.954	29.352	52.046	2.86 R
23	293.000	254.168	29.352	38.832	2.13 R
24	102.000	154.046	29.352	-52.046	-2.86 R

R denotes an observation with a large standardized residual.

Least Squares Means for FRAP (µmol TE / gRM)

Parts of fruit	Mean	SE Mean
Endocarp + seeds	35.50	14.100
Seeds	109.00	14.100
Peel	103.17	14.100
Endocarp	177.33	14.100
Whole fruit	115.50	14.100
Mesocarp	128.84	14.100
Temperature		
50	74.79	8.141
80	148.32	8.141
Pressure (bar)		
2	115.06	9.970
12	121.79	9.970
20	97.82	9.970
Parts of fruit*Temperature		
Endocarp + seeds 50	24.00	19.941
Endocarp + seeds 80	47.00	19.941
Seeds 50	74.33	19.941
Seeds 80	143.67	19.941
Peel 50	74.00	19.941
Peel 80	132.33	19.941

```
Endocarp     50            129.00   19.941
Endocarp     80            225.67   19.941
Whole fruit  50             73.00   19.941
Whole fruit  80            158.00   19.941
Mesocarp     50             74.42   19.941
Mesocarp     80            183.27   19.941
Parts of fruit*Pressure (bar)
Endocarp + seeds  2          32.50   24.422
Endocarp + seeds 12          39.50   24.422
Endocarp + seeds 20          34.50   24.422
Seeds         2              93.00   24.422
Seeds        12             121.00   24.422
Seeds        20             113.00   24.422
Peel          2              97.00   24.422
Peel         12             127.00   24.422
Peel         20              85.50   24.422
Endocarp      2             212.50   24.422
Endocarp     12             200.00   24.422
Endocarp     20             119.50   24.422
Whole fruit   2             110.50   24.422
Whole fruit  12             109.50   24.422
Whole fruit  20             126.50   24.422
Mesocarp      2             144.85   24.422
Mesocarp     12             133.76   24.422
Mesocarp     20             107.92   24.422
Temperature *Pressure (bar)
50            2              70.34   14.100
50           12              79.19   14.100
50           20              74.84   14.100
80            2             159.78   14.100
80           12             164.39   14.100
80           20             120.80   14.100
```

General Linear Model: DPPH (µmol T versus Parts of fruit, Temperature , ...

```
Factor            Type   Levels  Values
Parts of fruit    fixed       6  Endocarp + seeds, Seeds, Peel, Endocarp, Whole
                                 fruit, Mesocarp
Temperature (°C)  fixed       2  50, 80
Pressure (bar)    fixed       3  2, 12, 20

Analysis of Variance for DPPH (µmol TE / gRM), using Adjusted SS for Tests

Source                           DF   Seq SS   Adj SS  Adj MS      F      P
Parts of fruit                    5  31819.7  31819.7  6363.9  43.81  0.000
Temperature (°C)                  1   8697.4   8697.4  8697.4  59.88  0.000
Pressure (bar)                    2    781.3    781.3   390.7   2.69  0.116
Parts of fruit*Temperature (°C)   5   5259.3   5259.3  1051.9   7.24  0.004
Parts of fruit*Pressure (bar)    10   2064.8   2064.8   206.5   1.42  0.294
Temperature (°C)*Pressure (bar)   2    267.6    267.6   133.8   0.92  0.429
Error                            10   1452.5   1452.5   145.2
Total                            35  50342.6

S = 12.0518   R-Sq = 97.11%   R-Sq(adj) = 89.90%

Unusual Observations for DPPH (µmol TE / gRM)

     DPPH (µmol
Obs   TE / gRM)      Fit  SE Fit  Residual  St Resid
 20      25.000   41.811  10.242   -16.811     -2.65 R
 21      19.900    5.831  10.242    14.069      2.21 R
 23     142.000  125.189  10.242    16.811      2.65 R
 24      63.000   77.069  10.242   -14.069     -2.21 R

R denotes an observation with a large standardized residual.

Least Squares Means for DPPH (µmol TE / gRM)
```

Parts of fruit	Mean	SE Mean
Endocarp + seeds	14.433	4.920
Seeds	37.483	4.920
Peel	25.350	4.920
Endocarp	64.150	4.920
Whole fruit	72.150	4.920
Mesocarp	101.180	4.920
Temperature		
50	36.914	2.841
80	68.001	2.841
Pressure (bar)		
2	52.248	3.479
12	58.266	3.479
20	46.860	3.479
Parts of fruit*Temperature		
Endocarp + seeds 50	9.200	6.958
Endocarp + seeds 80	19.667	6.958
Seeds 50	34.667	6.958
Seeds 80	40.300	6.958
Peel 50	18.033	6.958
Peel 80	32.667	6.958
Endocarp 50	26.300	6.958
Endocarp 80	102.000	6.958
Whole fruit 50	53.333	6.958
Whole fruit 80	90.967	6.958
Mesocarp 50	79.954	6.958
Mesocarp 80	122.407	6.958
Parts of fruit*Pressure (bar)		
Endocarp + seeds 2	11.000	8.522
Endocarp + seeds 12	17.900	8.522
Endocarp + seeds 20	14.400	8.522
Seeds 2	26.450	8.522
Seeds 12	49.000	8.522
Seeds 20	37.000	8.522
Peel 2	24.850	8.522
Peel 12	30.450	8.522
Peel 20	20.750	8.522
Endocarp 2	67.500	8.522
Endocarp 12	83.500	8.522
Endocarp 20	41.450	8.522
Whole fruit 2	73.800	8.522
Whole fruit 12	67.200	8.522
Whole fruit 20	75.450	8.522
Mesocarp 2	109.886	8.522
Mesocarp 12	101.544	8.522
Mesocarp 20	92.110	8.522
Temperature *Pressure (bar)		
50 2	38.312	4.920
50 12	38.883	4.920
50 20	33.548	4.920
80 2	66.183	4.920
80 12	77.648	4.920
80 20	60.172	4.920

Appendix C

Appendix C contains additional information pertaining to **Chapter 5** which includes the analysis of variance (ANOVA) of the data generated for the experimental planning performed to optimize the extraction of genipap; adjustment routines and parameters adjusted by the spline model; and the diagrams obtained from the simulation performed in SuperPro Designer.

Table C.1: Analysis of variance (ANOVA).

General Linear Model: X0 (%) versus Solvents, Temperature , Pressure

```
Factor            Type   Levels  Values
Solvents          fixed       2  Water, Ethanol
Temperature (°C)  fixed       3  40, 50, 60
Pressure (MPa)    fixed       4  0, 2, 5, 8


Analysis of Variance for X0 (%), using Adjusted SS for Tests

Source                           DF  Seq SS  Adj SS  Adj MS     F      P
Solvents                          1  162.46  162.46  162.46  9.05  0.024
Temperature (°C)                  2   35.23   35.23   17.62  0.98  0.428
Pressure (MPa)                    3   25.33   25.33    8.44  0.47  0.714
Solvents*Temperature (°C)         2   36.47   36.47   18.24  1.02  0.417
Solvents*Pressure (MPa)           3   38.93   38.93   12.98  0.72  0.574
Temperature (°C)*Pressure (MPa)   6   57.35   57.35    9.56  0.53  0.769
Error                             6  107.73  107.73   17.95
Total                            23  463.50


S = 4.23732   R-Sq = 76.76%   R-Sq(adj) = 10.90%


Unusual Observations for X0 (%)

Obs   X0 (%)      Fit  SE Fit  Residual  St Resid
  4  33.2695  37.5936  3.6696   -4.3241     -2.04 R
 12  46.4906  42.1664  3.6696    4.3241      2.04 R

R denotes an observation with a large standardized residual.
```

General Linear Model: mg of genipin versus Solvents, Temperature, Pressure

```
Factor            Type   Levels  Values
Solvents          fixed       2  Water, Ethanol
Temperature (°C)  fixed       3  40, 50, 60
Pressure (MPa)    fixed       4  0, 2, 5, 8


Analysis of Variance for mg  genipin / g RM, using Adjusted SS for Tests

Source                           DF  Seq SS  Adj SS  Adj MS      F      P
Solvents                          1  4009.8  4009.8  4009.8  35.35  0.001
Temperature (°C)                  2    96.7    96.7    48.4   0.43  0.671
Pressure (MPa)                    3   496.0   496.0   165.3   1.46  0.317
Solvents*Temperature (°C)         2   641.8   641.8   320.9   2.83  0.136
Solvents*Pressure (MPa)           3   130.1   130.1    43.4   0.38  0.770
Temperature (°C)*Pressure (MPa)   6   296.1   296.1    49.3   0.43  0.833
Error                             6   680.6   680.6   113.4
Total                            23  6351.1
```

Table C.2: Programming routine used in SAS 9.2 (SAS Institute, Inc.) for the adjustment of experimental data of overall yield of the LPSE process to a spline of 3 straight lines.

```
/* ------------------------------------------------------------------ */
/* Departamento de Engenharia de Alimentos - DEA / Unicamp            */
/* Ajuste das curvas no SAS                                           */
/* Grazielle Náthia Neves - Campinas 30 de maio de 2017               */
/* ------------------------------------------------------------------ */
/* --[Cabeçalho]------------------------------------------------------ */
   Options NoDate NoNumber PS=100 LS=100 FormDLim='-';
   Title'Ensaio Cinetico 26 - Global Yield - Condicoes: Raw material = Genipap;
Pressure = 0.1 bar; Temperaure = 40°C; Solvent = H2O';
   FootNote;
/*----Digitação e leitura interna dos dados]------------------------- */
```

```
data E2NLIN;
       input tmin rend;
       AL1 = max(tmin-10,0);
       AL2 = max(tmin-25,0);
       Cards;
       0.5     2.549077292
       1       7.624961578
       2       10.11055431
       3       12.20654318
       4       16.92008647
       7       25.42244391
       10      32.64246144
       13      36.30688138
       16      39.01458038
       19      41.04834072
       25      43.79338594
       31      45.5129363
       37      46.6529505
       43      47.42476511
       55      48.48341349
       67      49.22440874
       79      49.71095883
       91      50.02427696
       103     50.40110412
       115     50.66686449
       127     50.98395243
;
Proc NLIN;
       Parms
       b0 = 3.40960
       b1 = 3.05014
       b2 = -2.26223
       b3 = -0.72759
       c1 = 10
       c2 = 25;
       AL1 = max(tmin - c1, 0);
       AL2 = max(tmin - c2, 0);
       Model rend = b0 + b1*tmin +
b2*AL1 + b3*AL2;
       Output out = a p = rendi r =
Mrend;

Proc print;
       Axis1 order = (0 to 55 by 5);
Proc gplot;
       Plot Mrend * rendi;
Proc gplot;
       Symbol1 value = diamond color =
black;
       Symbol2 value = star color =
blue;
       Plot rend*tmin/legend overlay
vaxis = axis1;
       Plot2 rendi*tmin/legend overlay
vaxis = axis1;
run;
```

```
data E1GNN;
       input tmin mext;
       AL1 = max(tmin-10,0);
       AL2 = max(tmin-25,0);
       Cards;
       0.5     2.549077292
       1       7.624961578
       2       10.11055431
       3       12.20654318
       4       16.92008647
       7       25.42244391
       10      32.64246144
       13      36.30688138
       16      39.01458038
       19      41.04834072
       25      43.79338594
       31      45.5129363
       37      46.6529505
       43      47.42476511
       55      48.48341349
       67      49.22440874
       79      49.71095883
       91      50.02427696
       103     50.40110412
       115     50.66686449
       127     50.98395243
;
Proc Reg;
       Model mext = tmin AL1 AL2;
       Output out = a p = mexthat r =
Mres;
Proc print;
       Axis1 order = (0 to 55 by 5);
Proc gplot;
       Plot Mres * mexthat;
Proc gplot;
       Symbol1 value = diamond color =
black;
       Symbol2 value = star color =
blue;
       Plot mext*tmin/legend overlay
vaxis = axis1;
       Plot2 mexthat*tmin/legend
overlay vaxis = axis1;
run;
```

Table C.3: Programming routine used in SAS 9.2 (SAS Institute, Inc.) for the adjustment of the experimental data of recovery of genipin of the process LPSE to a spline of 3 straight ones.

```
/* ---------------------------------------------------------------- */
/* Departamento de Engenharia de Alimentos - DEA / Unicamp          */
/* Ajuste das curvas no SAS                                         */
/* Grazielle Nathia Neves - Campinas 30 de maio de 2017             */
/* ---------------------------------------------------------------- */
/* --[Cabeçalho]----------------------------------------------------- */
   Options NoDate NoNumber PS=100 LS=100 FormDLim='-';
   Title'Ensaio Cinetico 26 - Genipin recovery - Condicoes: Raw material =
Genipap; Pressure = 0bar; Temperaure = 40oC; Solvent = H2O';
   FootNote;
/*----Digitação e leitura interna dos dados]------------------------- */
```

```
data E2NLIN;
       input tmin rend;
       AL1 = max(tmin-7,0);
       AL2 = max(tmin-25,0);
       Cards;
0.5     5.461630952
1       8.275574639
2       13.05042647
3       21.64575273
4       30.44026987
7       44.65734809
10      54.23859692
13      60.98317714
16      66.1288159
19      69.91986288
25      75.18074499
31      78.27014452
37      80.21748824
43      81.35808262
55      82.63836396
67      83.23955172
79      83.54706605
91      83.72637447
103     83.83263823
115     83.90817068
127     83.96515916
;
Proc NLIN;
       Parms
       b0 = 1.55297
       b1 = 6.71127
       b2 = -4.99458
       b3 = -1.65939
       c1 = 7
       c2 = 25;
       AL1 = max(tmin - c1, 0);
       AL2 = max(tmin - c2, 0);
       Model rend = b0 + b1*tmin +
b2*AL1 + b3*AL2;
       Output out = a p = rendi r =
Mrend;
Proc print;
       Axis1 order = (0 to 85 by 5);
Proc gplot;
       Plot Mrend * rendi;
Proc gplot;
       Symbol1 value = diamond color =
black;
       Symbol2 value = star color =
blue;
       Plot rend*tmin/legend overlay
vaxis = axis1;
       Plot2 rendi*tmin/legend overlay
vaxis = axis1;
run;
```

```
data E1GNN;
       input tmin mext;
       AL1 = max(tmin-7,0);
       AL2 = max(tmin-25,0);
       Cards;
0.5     5.461630952
1       8.275574639
2       13.05042647
3       21.64575273
4       30.44026987
7       44.65734809
10      54.23859692
13      60.98317714
16      66.1288159
19      69.91986288
25      75.18074499
31      78.27014452
37      80.21748824
43      81.35808262
55      82.63836396
67      83.23955172
79      83.54706605
91      83.72637447
103     83.83263823
115     83.90817068
127     83.96515916

;
Proc Reg;
       Model mext = tmin AL1 AL2;
       Output out = a p = mexthat r =
Mres;
Proc print;
       Axis1 order = (0 to 85 by 5);
Proc gplot;
       Plot Mres * mexthat;
Proc gplot;
       Symbol1 value = diamond color =
black;
       Symbol2 value = star color =
blue;
       Plot mext*tmin/legend overlay
vaxis = axis1;
       Plot2 mexthat*tmin/legend
overlay vaxis = axis1;
run;
```

Table C.4: Programming routine used in SAS 9.2 (SAS Institute, Inc.) for the adjustment of the experimental data of the overall yield of the Press + LPSE process to a 3-line spline.

```
/* ---------------------------------------------------------------- */
/* Departamento de Engenharia de Alimentos - DEA / Unicamp          */
/* Ajuste das curvas no SAS                                         */
/* Grazielle Náthia Neves - Campinas 30 de maio de 2017             */
/* ---------------------------------------------------------------- */
/* --[Cabeçalho]----------------------------------------------------- */
   Options NoDate NoNumber PS=100 LS=100 FormDLim='-';
   Title'Ensaio Cinetico 28 - Global Yield - Condicoes: Raw material =
Genipap; Pressure = 0bar; Temperaure = 40 °C; Solvent = H2O';
   FootNote;
/*----Digitação e leitura interna dos dados]------------------------- */
```

```
data E2NLIN;
       input tmin rend;
       AL1 = max(tmin-7,0);
       AL2 = max(tmin-25,0);
       Cards;
1.19   24.47960425
1.69   25.82647115
2.19   26.15750244
3.19   28.55717187
4.19   30.61119348
5.19   31.98277507
8.19   33.74208824
11.19  34.61904219
14.19  35.12630879
17.19  35.46578514
20.19  35.69985419
26.19  36.04220912
32.19  36.19945619
38.19  36.30203308
44.19  36.38943055
56.19  36.50989156
68.19  36.58320789
80.19  36.62349339
92.19  36.68006678
104.19 36.75427863
116.19 36.7936849
128.19 36.83727535
;
Proc NLIN;
       Parms
       b0 = 23.02479
       b1 = 1.62374
       b2 = -1.52429
       b3 = -0.09227
       c1 = 7
       c2 = 25;
       AL1 = max(tmin - c1, 0);
       AL2 = max(tmin - c2, 0);
       Model rend = b0 + b1*tmin +
b2*AL1 + b3*AL2;
       Output out = a p = rendi r =
Mrend;
Proc print;
       Axis1 order = (0 to 40 by 5);
Proc gplot;
       Plot Mrend * rendi;
Proc gplot;
       Symbol1 value = diamond color =
black;
       Symbol2 value = star color =
blue;
       Plot rend*tmin/legend overlay
vaxis = axis1;
       Plot2 rendi*tmin/legend overlay
vaxis = axis1;
run;
```

```
data E1GNN;
       input tmin mext;
       AL1 = max(tmin-7,0);
       AL2 = max(tmin-25,0);
       Cards;
1.19   24.47960425
1.69   25.82647115
2.19   26.15750244
3.19   28.55717187
4.19   30.61119348
5.19   31.98277507
8.19   33.74208824
11.19  34.61904219
14.19  35.12630879
17.19  35.46578514
20.19  35.69985419
26.19  36.04220912
32.19  36.19945619
38.19  36.30203308
44.19  36.38943055
56.19  36.50989156
68.19  36.58320789
80.19  36.62349339
92.19  36.68006678
104.19 36.75427863
116.19 36.7936849
128.19 36.83727535

;
Proc Reg;
       Model mext = tmin AL1 AL2;
       Output out = a p = mexthat r =
Mres;
Proc print;
       Axis1 order = (0 to 40 by 5);
Proc gplot;
       Plot Mres * mexthat;
Proc gplot;
       Symbol1 value = diamond color =
black;
       Symbol2 value = star color =
blue;
       Plot mext*tmin/legend overlay
vaxis = axis1;
       Plot2 mexthat*tmin/legend
overlay vaxis = axis1;
run;
```

Table C.5: Programming routine used in SAS 9.2 (SAS Institute, Inc.) to adjust the genipin recovery data from the Press + LPSE process to a 3-line spline.

```
/* ---------------------------------------------------------------- */
/* Departamento de Engenharia de Alimentos - DEA / Unicamp          */
/* Ajuste das curvas no SAS                                         */
/* Grazielle Náthia Neves - Campinas 30 de maio de 2017             */
/* ---------------------------------------------------------------- */
/* --[Cabeçalho]----------------------------------------------------- */
   Options NoDate NoNumber PS=100 LS=100 FormDLim='-';
   Title'Ensaio Cinetico 28 - Genipin recovery - Condicoes: Raw material =
Genipap; Pressure = 0bar; Temperaure = 40 °C; Solvent = H2O';
   FootNote;
/*----Digitação e leitura interna dos dados]------------------------- */
```

```
data E1GNN;
       input tmin mext;
       AL1 = max(tmin-7,0);
       AL2 = max(tmin-25,0);
       Cards;
1.19   43.43532195
1.69   46.30908459
2.19   49.04750869
3.19   54.87651135
4.19   59.97712712
5.19   63.68076523
8.19   68.37468282
11.19  70.75620041
14.19  72.2283351
17.19  73.17354955
20.19  73.84972442
26.19  74.65402807
32.19  75.13233494
38.19  75.42321987
44.19  75.62460233
56.19  75.86773541
68.19  75.99756287
80.19  76.08348731
92.19  76.1400446
104.19 76.18146698
116.19 76.21259399
128.19 76.21270998
;
Proc Reg;
       Model mext = tmin AL1 AL2;
       Output out = a p = mexthat r =
Mres;
Proc print;
       Axis1 order = (0 to 80 by 5);
Proc gplot;
       Plot Mres * mexthat;
Proc gplot;
       Symbol1 value = diamond color =
black;
       Symbol2 value = star color =
blue;
       Plot mext*tmin/legend overlay
vaxis = axis1;
       Plot2 mexthat*tmin/legend
overlay vaxis = axis1;
run;
```

```
data E2NLIN;
       input tmin rend;
       AL1 = max(tmin-7,0);
       AL2 = max(tmin-25,0);
       Cards;
1.19   26.64243516
1.69   28.28091937
2.19   29.74754981
3.19   32.12539528
4.19   34.05877495
5.19   35.48970202
8.19   37.6703257
11.19  38.63644875
14.19  39.24196247
17.19  39.60288907
20.19  39.85418997
26.19  40.16204561
32.19  40.36517546
38.19  40.50253898
44.19  40.59014916
56.19  40.737262
68.19  40.86814978
80.19  41.02098569
92.19  41.13638724
104.19 41.17065459
116.19 41.23245701
128.19 41.28128059
;
Proc NLIN;
       Parms
       b0 = 25.41005
       b1 = 1.86465
       b2 = -1.76095
       b3 = -0.09312
       c1 = 7
       c2 = 25;
       AL1 = max(tmin - c1, 0);
       AL2 = max(tmin - c2, 0);
       Model rend = b0 + b1*tmin +
b2*AL1 + b3*AL2;
       Output out = a p = rendi r =
Mrend;
Proc print;
       Axis1 order = (0 to 45 by 5);
Proc gplot;
       Plot Mrend * rendi;
Proc gplot;
       Symbol1 value = diamond color =
black;
       Symbol2 value = star color =
blue;
       Plot rend*tmin/legend overlay
vaxis = axis1;
       Plot2 rendi*tmin/legend overlay
vaxis = axis1;
run;
```

Table C.6: Project indices of the LPSE process obtained by SuperPro Designer

LPSE	Scenario	Selling Price (US$/kg)	GM (%)	ROI (%)	Payback time (year)	IRR (%)	NPV (US$) (at 7% interest)
Raw material value = 1.42 US$/Kg							
2 x 100 L	1	50	-2.13	7.79	12.84	N/A	-422,000
	2	100	48.94	28.83	3.47	22.42	1,121,000
	3	150	65.96	49.57	2.02	38.05	2,647,000
	4	200	74.47	70.32	1.42	51.17	4,173,000
	5	250	79.57	91.07	1.1	62.27	5,682,000
2x 50 L	6	50	-20.62	2.94	34.07	N/A	-479,000
	7	100	39.69	21.5	4.65	15.7	368,000
	8	150	59.79	37.82	2.64	29.61	1,131,000
	9	200	69.85	54.14	1.85	41.02	1,894,000
	10	250	75.88	70.47	1.42	51.17	2,657,000
2x 10 L	11	50	-96.63	-6.12	N/A	N/A	-321,000
	12	100	1.69	8.88	11.27	N/A	-83,000
	13	150	34.46	17.99	5.56	11.8	70,000
	14	200	50.84	27.11	3.69	20.55	222,000
	15	250	60.67	36.23	2.76	28.05	375,000
Raw material value = 7.89 US$/Kg							
2 x 100 L	16	50	-168.24	-47.18	N/A	N/A	-4,576,000
	17	100	-34.12	-14.32	N/A	N/A	-2,067,000
	18	150	10.59	14.36	6.96	8.36	87,000
	19	200	32.94	34.08	2.93	27.11	1,613,000
	20	250	46.35	53.79	1.86	41.48	3,139,000
2x 50 L	21	50	-186.73	-40.58	N/A	N/A	-2,566,000
	22	100	-43.36	-14.45	N/A	N/A	-1,312,000
	23	150	4.42	10.29	9.72	3.05	-143,000
	24	200	28.32	25.96	3.85	20.08	613,000
	25	250	42.65	41.64	2.4	32.73	1,376,000
2x 10 L	26	50	-262.74	-30.66	N/A	N/A	-740,000
	27	100	-81.37	-15.8	N/A	N/A	-490,000
	28	150	-20.91	-0.94	N/A	N/A	-239,000
	29	200	9.32	11.7	8.55	4.77	-30,000
	30	250	27.45	20.61	4.85	14.77	119,000

NA: Not applicable; ROI: Return on investment; IRR: Internal rate of return after taxes; NPV: Net present value.

Table C.7- Project indices of the Press+LPSE process obtained by SuperPro Designer

Press + LPSE	Scenario	Selling Price (US$/kg)	GM (%)	ROI (%)	Payback time (year)	IRR (%)	NPV (US$) (at 7% interest)
Raw material value = 1.42 US$/Kg							
2 x 100 L	31	50	5.85	9.97	10.03	2.11	-272,000
	32	100	52.92	35.34	2.83	27.89	1,713,000
	33	150	68.62	60.72	1.65	45.39	3,706,000
	34	200	76.46	86.09	1.16	59.77	5,685,000
	35	250	81.17	111.46	0.9	72.11	7,651,000
2x 50 L	36	50	-9.2	5.47	18.28	N/A	-395,000
	37	100	45.4	26.58	3.76	20.55	651,000
	38	150	63.6	46.47	2.15	35.86	1,647,000
	39	200	72.7	66.36	1.51	48.83	2,644,000
	40	250	78.16	86.25	1.16	59.77	3,632,000
2x 10 L	41	50	-63.47	-3.19	N/A	N/A	-289,000
	42	100	18.27	12.62	7.92	6.02	-14,000
	43	150	45.51	23.74	4.21	17.89	181,000
	44	200	59.13	34.85	2.87	27.27	380,000
	45	250	67.31	45.96	2.18	35.55	579,000
Raw material value = 7.89 US$/Kg							
2 x 100 L	46	50	-185.01	-64.97	N/A	N/A	-6,453,000
	47	100	-42.5	-25.58	N/A	N/A	-3,176,000
	48	150	5	11.45	8.73	5.23	-115,000
	49	200	28.75	35.09	2.85	28.2	1,856,000
	50	250	43	58.72	1.7	45.08	3,849,000
2x 50 L	51	50	-200.05	-54.66	N/A	N/A	-3,523,000
	52	100	-50.03	-23.31	N/A	N/A	-1,884,000
	53	150	-0.02	8.04	12.44	N/A	-275,000
	54	200	24.99	26.85	3.72	21.02	722,000
	55	250	39.99	45.66	2.19	35.86	1,719,000
2x 10 L	56	50	-254.32	-37.34	N/A	N/A	-919,000
	57	100	-77.16	-19.39	N/A	N/A	-591,000
	58	150	-18.11	-1.45	N/A	N/A	-263,000
	59	200	11.42	13.22	7.57	7.11	3,000
	60	250	29.14	23.98	4.17	18.2	195,000

NA: Not applicable; ROI: Return on investment; IRR: Internal rate of return after taxes; NPV: Net present value.

Figure 1F contains permission to use the corresponding article to chapter 5.

Figure 1F – Copyright Chapter 5.

Appendix D

Appendix D contains additional information pertaining to **Chapter 6** which includes the analysis of variance (ANOVA) of the data generated for the experimental planning performed to optimize the extraction of the genipap oil.

Table D.1: Analysis of variance (ANOVA) for X0.

General Linear Model: X0 (%) versus Temperature (°C); Pressure (MPa)

```
Factor            Type   Levels  Values
Temperature (°C)  fixed       2  40; 60
Pressure (MPa)    fixed       5  15; 20; 25; 30; 35


Analysis of Variance for X0 (%), using Adjusted SS for Tests

Source                           DF   Seq SS   Adj SS   Adj MS      F
P
Temperature (°C)                  1  0,03877  0,03877  0,03877   2,23
0,167
Pressure (MPa)                    4  5,76254  5,76254  1,44063  82,71
0,000
Temperature (°C)*Pressure (MPa)   4  1,27846  1,27846  0,31961  18,35
0,000
Error                            10  0,17418  0,17418  0,01742
Total                            19  7,25395


S = 0,131979   R-Sq = 97,60%   R-Sq(adj) = 95,44%


Least Squares Means for X0 (%)

Temperature                 Mean  SE Mean
40                         3,917  0,04174
60                         3,829  0,04174
Pressure (MP
15                         3,005  0,06599
20                         3,533  0,06599
25                         4,037  0,06599
30                         4,381  0,06599
35                         4,409  0,06599
Temperature *Pressure (MP
40           15            3,481  0,09332
40           20            3,708  0,09332
40           25            3,967  0,09332
40           30            4,172  0,09332
40           35            4,256  0,09332
60           15            2,529  0,09332
60           20            3,358  0,09332
60           25            4,106  0,09332
60           30            4,589  0,09332
60           35            4,562  0,09332
```

Table D.2: Analysis of variance (ANOVA) for palmitic acid.

General Linear Model: Palmitic aci versus Temperature ; Pressure (MP

```
Factor            Type   Levels  Values
Temperature (°C)  fixed       2  40; 60
Pressure (MPa)    fixed       5  15; 20; 25; 30; 35


Analysis of Variance for Palmitic acid (mg/g oil), using Adjusted SS for
Tests

Source                           DF  Seq SS  Adj SS  Adj MS     F      P
Temperature (°C)                  1    2,66    2,66    2,66  0,23  0,645
Pressure (MPa)                    4   57,75   57,75   14,44  1,22  0,362
Temperature (°C)*Pressure (MPa)   4   33,36   33,36    8,34  0,70  0,607
Error                            10  118,37  118,37   11,84
Total                            19  212,15


S = 3,44057   R-Sq = 44,20%   R-Sq(adj) = 0,00%


Unusual Observations for Palmitic acid (mg/g oil)

       Palmitic
     acid (mg/g
Obs        oil)      Fit  SE Fit  Residual  St Resid
  9     30,0000  24,0000  2,4328    6,0000      2,47 R
 19     18,0000  24,0000  2,4328   -6,0000     -2,47 R

R denotes an observation with a large standardized residual.


Least Squares Means for Palmitic acid (mg/g oil)

Temperature                 Mean  SE Mean
40                         25,15    1,088
60                         24,42    1,088
Pressure (MP
15                         24,25    1,720
20                         24,68    1,720
25                         26,00    1,720
30                         22,00    1,720
35                         27,00    1,720
Temperature *Pressure (MP
40           15            25,75    2,433
40           20            26,00    2,433
40           25            26,00    2,433
40           30            20,00    2,433
40           35            28,00    2,433
60           15            22,75    2,433
60           20            23,35    2,433
60           25            26,00    2,433
60           30            24,00    2,433
60           35            26,00    2,433
```

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Table D.3: Analysis of variance (ANOVA) for stearic acid.

General Linear Model: Stearic acid versus Temperature ; Pressure (MP

Factor	Type	Levels	Values
Temperature (˚C)	fixed	2	40; 60
Pressure (MPa)	fixed	5	15; 20; 25; 30; 35

Analysis of Variance for Stearic acid (mg/g oil), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Temperature (˚C)	1	11,705	11,705	11,705	2,59	0,138
Pressure (MPa)	4	5,722	5,722	1,431	0,32	0,860
Temperature (˚C)*Pressure (MPa)	4	4,698	4,698	1,175	0,26	0,897
Error	10	45,105	45,105	4,511		
Total	19	67,230				

S = 2,12379 R-Sq = 32,91% R-Sq(adj) = 0,00%

Least Squares Means for Stearic acid (mg/g oil)

Temperature		Mean	SE Mean
40		15,77	0,6716
60		14,24	0,6716
Pressure (MP			
15		14,40	1,0619
20		14,83	1,0619
25		14,80	1,0619
30		15,00	1,0619
35		16,00	1,0619
Temperature *Pressure (MP			
40	15	14,80	1,5017
40	20	16,05	1,5017
40	25	16,00	1,5017
40	30	16,00	1,5017
40	35	16,00	1,5017
60	15	14,00	1,5017
60	20	13,60	1,5017
60	25	13,60	1,5017
60	30	14,00	1,5017
60	35	16,00	1,5017

Table D.4: Analysis of variance (ANOVA) linoleic acid.

General Linear Model: Linoleic aci versus Temperature ; Pressure (MP

```
Factor            Type   Levels  Values
Temperature (°C)  fixed       2  40; 60
Pressure (MPa)    fixed       5  15; 20; 25; 30; 35


Analysis of Variance for Linoleic acid (mg/g oil), using Adjusted SS for
Tests

Source                           DF   Seq SS   Adj SS   Adj MS      F      P
Temperature (°C)                  1    897,8    897,8    897,8   5,34  0,043
Pressure (MPa)                    4  43071,2  43071,2  10767,8  64,02  0,000
Temperature (°C)*Pressure (MPa)   4   5071,2   5071,2   1267,8   7,54  0,005
Error                            10   1682,0   1682,0    168,2
Total                            19  50722,2


S = 12,9692   R-Sq = 96,68%   R-Sq(adj) = 93,70%


Unusual Observations for Linoleic acid (mg/g oil)

      Linoleic
    acid (mg/g
Obs        oil)      Fit  SE Fit  Residual  St Resid
  5     332,000  312,000   9,171    20,000      2,18 R
 15     292,000  312,000   9,171   -20,000     -2,18 R

R denotes an observation with a large standardized residual.


Least Squares Means for Linoleic acid (mg/g oil)

Temperature                 Mean  SE Mean
40                         269,4    4,101
60                         256,0    4,101
Pressure (MP
15                         171,5    6,485
20                         272,0    6,485
25                         282,0    6,485
30                         290,0    6,485
35                         298,0    6,485
Temperature *Pressure (MP
40           15            147,0    9,171
40           20            292,0    9,171
40           25            292,0    9,171
40           30            304,0    9,171
40           35            312,0    9,171
60           15            196,0    9,171
60           20            252,0    9,171
60           25            272,0    9,171
60           30            276,0    9,171
60           35            284,0    9,171
```

Table D.5: Analysis of variance (ANOVA) for linolenic acid.

General Linear Model: Linolenic ac versus Temperature ; Pressure (MP

```
Factor            Type   Levels  Values
Temperature (°C)  fixed       2  40; 60
Pressure (MPa)    fixed       5  15; 20; 25; 30; 35


Analysis of Variance for Linolenic acid (mg/g oil), using Adjusted SS for
Tests

Source                           DF  Seq SS  Adj SS  Adj MS      F      P
Temperature (°C)                  1  200,98  200,98  200,98  12,06  0,006
Pressure (MPa)                    4  192,67  192,67   48,17   2,89  0,079
Temperature (°C)*Pressure (MPa)   4  125,71  125,71   31,43   1,89  0,189
Error                            10  166,58  166,58   16,66
Total                            19  685,94


S = 4,08142   R-Sq = 75,72%   R-Sq(adj) = 53,86%


Unusual Observations for Linolenic acid (mg/g oil)

      Linolenic
     acid (mg/g
Obs        oil)      Fit  SE Fit  Residual  St Resid
  8     52,0000  46,0000  2,8860    6,0000      2,08 R
 18     40,0000  46,0000  2,8860   -6,0000     -2,08 R

R denotes an observation with a large standardized residual.


Least Squares Means for Linolenic acid (mg/g oil)

Temperature                 Mean  SE Mean
40                         50,70    1,291
60                         44,36    1,291
Pressure (MP
15                         42,00    2,041
20                         46,90    2,041
25                         48,00    2,041
30                         49,75    2,041
35                         51,00    2,041
Temperature *Pressure (MP
40           15            50,00    2,886
40           20            50,00    2,886
40           25            50,00    2,886
40           30            51,50    2,886
40           35            52,00    2,886
60           15            34,00    2,886
60           20            43,80    2,886
60           25            46,00    2,886
60           30            48,00    2,886
60           35            50,00    2,886
```

Appendix E contains the SISGEN certificate.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão

Cadastro nº ADD59FD

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**ADD59FD**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Genipa americana

Título da Atividade:	**APLICAÇÃO DO CONCEITO DE BIORREFINARIA NA OBTENÇÃO DO CORANTE AZUL NATURAL DO JENIPAPO**

Equipe

Grazielle Náthia Neves	**UNICAMP**
Maria Angela de Almeida Meireles Petenate	**Universidade Estadual de Campinas**

Data do Cadastro:	**03/07/2018 17:28:50**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético

Situação cadastral conforme consulta ao SisGen em **17:08** de **05/02/2019**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN